EXPERIMENTAL CELL RESEARCH

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STUDIES ON THE ACTION OF THE "FUCUS FERTILIZATION INHIBITOR" ON SEA URCHIN EGG AND SPERM

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In different sea urchin species Runnström and Wicklund (10) found an inhibition of the membrane elevation when the fertilization occurred in presence of dissolved jelly coat substances from the same material.

In view of the similar properties of jelly coat substance and heparin this latter substance was also tested and found to produce an inhibition. Harding (2) extended this study to other sea urchin species and substances. He found one of the commercial preparations of heparin to be very active in preventing the membrane elevation. He also tested a crude preparation of "fucoidin" which was prepared in this laboratory by Dr. Vasseur. This preparation proved to be an active inhibitor of the membrane elevation. However, the inhibitory action could rapidly be removed by addition of sodium periodate, see also (8, 13). The more potent inhibitors not only suppressed the normal membrane formation but—in sufficient concentrations—they even blocked the fertilization completely. It seemed particularly promising to continue the work with the inhibitor from *Fucus vesiculosus*. According to further observations carried out by Vasseur *et al.* (12) the pure fucoidin is inactive as an inhibitor of the fertilization. The inhibition in Harding's experiments was due to some other non dialyzable substance present in the crude extract. Vasseur *et al.* therefore proceeded to a purification of the active agent which we give the provisional name "fertilization inhibitor" from *Fucus*. In agreement with the system previously adopted (4) the abbreviation FeInh[Fu] is used.

By a rather complicated fractionation with alcohol and finally with lead acetate more potent preparations were obtained. The antihyaluronidase action of FeInh[Fu] served as a test during the preparation and it was shown that the antihyaluronidase activity and the inhibitory action in fertilization run strictly parallel (12). The chemical nature of the inhabitor is, however, still unknown.

Recently Wicklund (13) continued the studies on FeInh[Fu] and a number of other inhibitors of fertilization. She contended that FeInh[Fu] and

other similar inhibitors are analogues of a natural inhibitor present in the egg cortex. In sufficient concentrations even the dissolved jelly coat substance is able to block the fertilization completely (13 p. 485).

MATERIAL AND METHODS

The sea urchins *Paracentrotus lividus* and *Arbacia lixula* served as material in the part of the work carried out in Naples. *Psammechinus miliaris* was used in experiments carried out in Kristineberg, the Zoological Station at the west coast of Sweden. The procedures of preparing the egg and sperm material were the customary, see for example Harding (2).

Two preparations of FeInh[Fu] were available: one, B6, represents the crude fucoidin preparation, where, as follows from the introduction, the impurities contained the active factor. The second preparation SupC_4I was considerably more purified, see above; it is active in lower concentrations than the former preparation. The activity depends, however, not only on the preparations but also on the egg material and its pretreatment. Some further information regarding methods is given below.

RESULTS

The action of FeInh[Fu] on the jelly coat

If eggs were taken directly from the ovary they often had a rather condensed jelly coat that slowly swelled in sea water. If, however, the eggs were immersed in sea water with a certain concentration of FeInh[Fu] the swelling of the jelly coat was strongly inhibited.

The following is an example from eggs of *Arbacia lixula* (Naples, 30.III.1953). Eggs with condensed jelly coat were transferred to the following test media (pH 8.0):

a. 2 ml sea water;
b. 2 ml 0.02 per cent FeInh[Fu] B6 in sea water.

After 30–40 minutes the jelly coat had a thickness of 25–50 μ in *a*, whereas the thickness in *b* was only about 6–12 μ. By crowding the eggs and measuring the distance between the eggs by means of a screw micrometer attached to the ocular of the microscope a rough estimation of the thickness of the jelly coat was possible. In eggs with condensed jelly coat the outline of the jelly coat could be directly distinguished.

A drop of egg suspension was transferred to a slide. This was made ca. two minutes after suspending the eggs in the test medium *a*. No essential swelling of the jelly coat had occurred as yet. A drop of a heavy sperm suspension (1 drop "dry sperm" to 2 ml sea water) was added to the drop of egg suspension and a cover slip with plasticin supports was mounted over the mixture of egg and sperm suspension. The same kind of observations were now made as those previously described by Hartmann and Schartau, see Hartmann (3) p. 389, Fig. 245.

A strong swelling of the jelly coat occurred. After 1–2 minutes the spermatozoa seemed to swim around in the jelly coat without any restraint. Often the spermatozoa penetrated below the jelly coat and exhibited very lively movement in a space between the jelly and the cytoplasmic surface. By a peculiar co-ordination of the movements of the spermatozoa the egg was brought into a rotation inside the jelly coat.

Gradually the jelly coat seemed to dissolve under the influence of the sperm. Often the partially dissolved jelly coat was removed from the egg and thereafter completely faded away. Suspended fragments of jelly were also vigorously attacked by the spermatozoa until they dissolved.

As was observed by Hartmann (3) the sperm liquor alone has also a dissolving action on the jelly coat. This was confirmed, see (4). It must remain an open question whether the dissolution of the jelly is due only to the sperm liquor or whether the spermatozoa also carry the active agent. If this is not the case, the spermatozoa probably contribute by mechanical action to the dispersion of the jelly coat substance.

In a similar way eggs from suspension *b* were heavily inseminated on a slide and observed under cover slip. For several minutes the spermatozoa were not able to penetrate into the jelly coat, although the movements of the spermatozoa obviously were enhanced in presence of FeInh[Fu], see (13). The whole egg surrounded by the tough jelly coat was brought into rotation under the impact of the co-ordinated motion of the spermatozoa. In the course of some minutes single spermatozoa penetrated into the jelly coat, but even after 90 minutes many spermatozoa stuck to the periphery of the jelly coat. The free spermatozoa still exhibited lively movements.

Five minutes after the mixing of the egg and spermatozoa a detached fragment of the jelly coat was discovered in the preparation. This was heavily attacked by a great number of spermatozoa, but it did not swell and fade as in *a*. Even after 90 minutes no perceptible change occurred in the fragment.

After 160 minutes the eggs in *b* were transferred to normal sea water and inseminated with a fresh dose of spermatozoa (1 drop of dry sperm to 5 ml sea water; from this suspension one drop was added pro ml egg suspension). There was no obvious swelling of the jelly coat but nevertheless a fertilization with a low adhering membrane occurred.

Figs. 1 and 2 illustrate the different swelling of the jelly coat of eggs of *Arbacia lixula* kept in sea water (*a*) and in 0.005 per cent FeInh[Fu] Sup C_4I in sea water (*b*). In *a* almost 100 per cent of the eggs were fertilized; conversely in *b* only very few of the eggs were fertilized upon insemination. In general there was a considerable interval between insemination and fertilization. The photos were taken 4.5 hours after insemination. In both Figs. 1 and 2 spermatozoa are visible in the jelly and their different distribution around the egg visualizes the different degree of swelling of the jelly coats in the two cases. It is also obvious that the egg of Fig. 2 is delayed in its development in comparison with that of Fig. 1. The cleavage of the former is rather abnormal, the cells are closely packed and the outline of the whole egg irregular.

Influence of the fertilization inhibitor from Fucus on the agglutination of the spermatozoa by jelly solution

FeInh[Fu] has in itself no agglutinating action on the spermatozoa. Neither does it prevent the agglutination of the spermatozoa in presence of dissolved jelly. The agglutination commences as in the absence of the FeInh[Fu]. During the reversal of the agglutination, however, there are certain pronounced differences.

The jelly coats were removed from *Paracentrotus* eggs, dialyzed against distilled water and freeze-dried. A 0.36 per cent solution was made up in sea water and centrifuged to remove some insoluble flakes. The remaining solution was used in dilutions 1:4 and 1:20.

A drop of the solution was brought between slide and cover slip and a second drop containing a strong suspension of *Paracentrotus* sperm (1 ml sea water + 1 drop dry sperm) was added (7). By a light pressure on the cover slip with a needle the drops were brought to fusion and observations were made on the behaviour of the spermatozoa. Both dilutions of the jelly solution gave an immediate strong head agglutination in the border between the fusing drops. After ca 35 seconds the head agglutination ceased. For another 10–20 seconds the spermatozoa were kept together in spherical groups but without being attached to each other as if they were sticking to an invisible substrate. In the present observations two different stages could thus be distinguished with respect to the behavior of the spermatozoa: that of head agglutination and that of more diffuse aggregation. Addition of FeInh[Fu] did not change the first phase but prolonged or stabilized the second.

In one experiment (Naples 28.IV.1953) the time course in 1/20 jelly solution was the following:

10 seconds, head agglutination visible;

27 seconds, the agglutinates changed into loose aggregates;

55 seconds, the aggregates dissolved; lively movements of single spermatozoa; see Fig. 3.

In a parallel test the medium contained the same concentration of jelly but also 0.06 per cent FeInh[Fu] Sup C_4 I:

Immediate strong head agglutination. After ca. 30 seconds looser aggregates appeared. These were not dissolved but often fused into larger more irregular aggregates; see Fig. 4.

A third test with 0.06 per cent FeInh[Fu] alone gave no agglutination of the spermatozoa.

Similar results as those reported were obtained also with sperm and egg water of *Arbacia lixula.*

The effect of FeInh[Fu] on eggs pretreated with sodium periodate

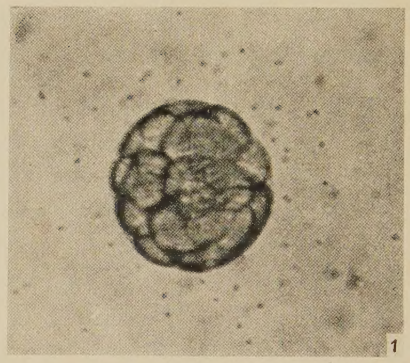
The effect of sodium periodate on the sea urchin egg has been subject to recent studies (5, 6, 8, 9). It is known that addition of periodate improves the fertilization of eggs pretreated with this reagent in concentrations $< 10^{-4}$ M. In this study the eggs were subjected to $4 \cdot 10^{-5}$ M periodate which in itself caused no activation. The egg material (*Paracentrotus lividus*) was selected so that the improvement of the fertilization was not very pronounced, i.e. even without pretreatment with periodate the eggs showed membrane elevation and normal development to the pluteus stage. Nevertheless the non pretreated eggs (suspension C) reacted more intensely against FeInh[Fu] than the periodate treated eggs (suspension PJ), see Table I.

TABLE I

Paracentrotus lividus, Naples, 27.IV.1953. The eggs of one female divided into two parts: Suspension C, 50 ml egg suspension in sea water; Suspension PJ, to 50 ml egg suspension 0.2 ml sodium periodate in dist. water was added (final conc. of periodate: 4.10^{-5} M). Eggs from suspension C and suspension PJ were transferred to dishes with diluted fertilization inhibitor from *Fucus*, preparation Sup C_4 I (Vasseur). Before the transfer the eggs from the suspensions C and PJ were washed twice in sea water. They were inseminated 9–10 minutes after the transfer.

The eggs were examined 20–30 minutes after the insemination. The percentage figures are based on counts of 100–120 eggs in each case.

Concentration of FeInh[Fu]	I. Eggs transferred from suspension C	II. Eggs transferred from suspension PJ
a. 0 per cent	> 98 per cent fertilized eggs. Jelly coat swollen, membrane somewhat asymmetrically elevated.	100 per cent fertilized eggs. Jelly coat swollen. Membrane more regular than in aI.
b. 0.00075 per cent	> 98 per cent fertilized. The eggs of more irregular shape than in *a.*	100 per cent fertilized eggs. Membrane low but uniformly elevated. Normal appearance of the egg.
c. 0.0015 per cent	31 per cent fertilized eggs, membrane low or asymmetrically elevated.	100 per cent fertilized eggs. Membrane low but uniformly elevated. Normal appearance of the eggs.
d. 0.003 per cent	4 per cent fertilized, no membrane elevation.	74 per cent fertilized.
e. 0.006 per cent	0 per cent fertilized egg. Jelly coat condensed with spermatozoa sticking to the peripheral part.	0 per cent fertilization. Jelly coat swollen.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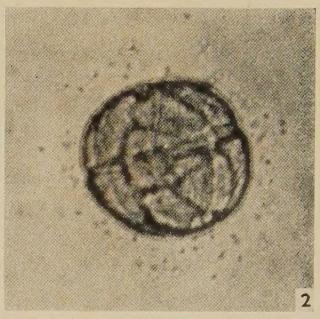
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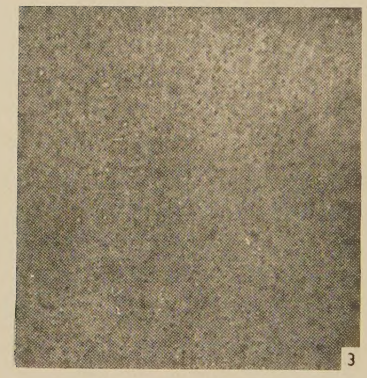
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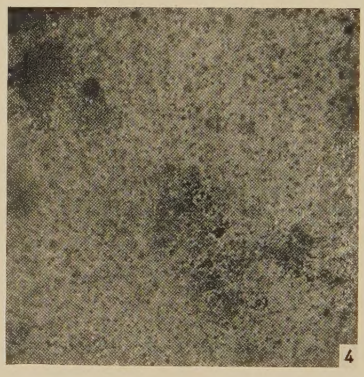
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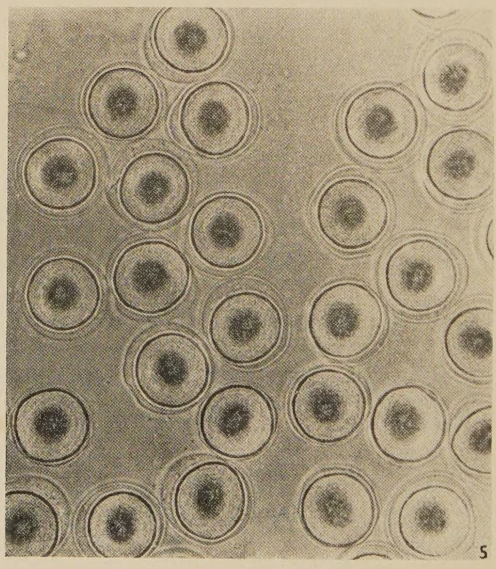
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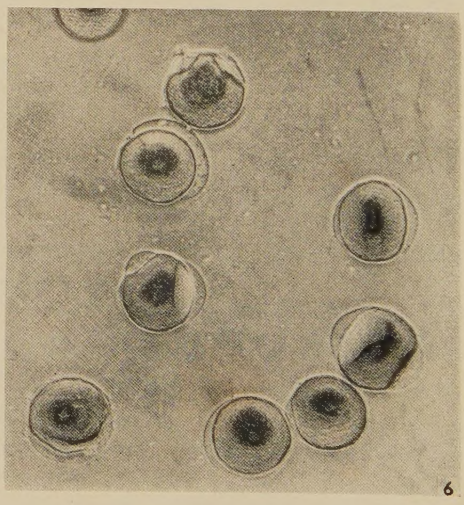
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After five hours it was found that the percentage of fertilized eggs had increased to about 11 per cent in sample I*e* and to 33 per cent in I*d*. (I*e* and I*d* non periodate pretreated eggs transferred to 0.006 and 0.003 per cent FuFeI respectively, see Table I.) There was a considerable scatter with respect to stages (2–8-cell in I*e*, 2–16-cell in I*d*). The scatter and early stage of cleavage in I*e* and I*d* were due to a delayed fertilization. This was possible because of the capacity of FeInh[Fu] to prolong the fertilizing life of the spermatozoa (13). In II*e* the increase in percentage fertilized was not considerable whereas in II*d* 100 per cent of the eggs were in cleavage of a rather regular and uniform type.

The jelly coat was more swollen in the periodate pretreated test media. Furthermore the cleavage was more advanced and uniform than in the non pretreated eggs. This held already for the controls; 16-, 32- and 64-cell stages were found in I*a*, whereas in II*a* 32- and particularly 64-cell stages prevailed. In the strongest dilution of FeInh[Fu] the non pretreated eggs (I*b*) contained 2–32-cell stages, but the periodate pretreated eggs (II*b*) mainly 32-64-cell stages. A certain inhibition and delay prevailed thus in I*b*, but this delay was much less pronounced in II*b*.

The development was followed in the different test media included in Table I. After 40–42 hours plutei were found to be present in the controls (I *a*, II *a*). In the samples *e* only very few undifferentiated larvae were present. Conversely numerous swimming larvae were found in *c* and *d*. These had all a radially symmetrical type. The radial symmetry is expressed in the arrangement of the cells of the primary mesenchyme cells which fail to concentrate to the two bilaterally arranged groups. As a consequence multiple radially arranged triradiate skeleton spicules appear. These do not differentiate in normal way, but retain a simple structure. The intestin remains straight. The anterior part of the ectoderm differentiates often to a lobe with high cylindrical epithelium. To varying extent this lobe is separated by a constriction from the rest of the ectoderm. A ciliary band with cylindrical epithelium surrounds the truncate anal field forming the posterior end of

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Fig. 1. Egg of *Arbacia lixula* inseminated in sea water; 4½ hours after fertilization. Fertilization membrane adhering to the egg surface. Spermatozoa present in the swollen jelly coat.
Fig. 2. Egg of *Arbacia lixula* inseminated in sea water in presence of 0.005 per cent fertilization inhibitor from *Fucus*, preparation Sup C_4 I. Condensed jelly coat with spermatozoa. Irregular cleavage.
Fig. 3. Spermatozoa of *Paracentrotus lividus* dispersed after a previous head agglutination induced by a jelly coat solution in sea water.
Fig. 4. Spermatozoa of *Paracentrotus* partially united to loose aggregates after previous head agglutination induced by a jelly coat solution with addition of 0.06 per cent fertilization inhibitor from *Fucus* (preparation Sup C_4 I).
Fig. 5. Eggs of *Paracentrotus lividus* deprived of their jelly coats inseminated in sea water. Phase contrast.
Fig. 6. Eggs of *Paracentrotus lividus* deprived of their jelly coats, inseminated in presence of 0.002 per cent fertilization inhibitor from *Fucus* (preparation Sup C_4 I). Phase contrast.

these larvae. When swimming they rotate around their anteroposterior axis. The reader is referred to Gustafson and Sävhagen (1, Fig. 1)[1], see also (8, 11).

Only one of the test media with FeInh[Fu] presented bilateral plutei, *viz.* II*b*, whereas the larvae in I*b* were of the radially symmetrical type. This seems to indicate that the periodate pretreatment has made the larvae more resistant to the "radialising" effect of FeInh[Fu] when dilute concentrations of the inhibitor is used.

Effect of the fertilization inhibitor from Fucus on eggs deprived of their jelly coats

The experiments reported in the previous section were continued and considerably extended by experiments carried out in the season 1954. The previous results (Table I) were confirmed. Particular attention was paid to the question whether FeInh[Fu] acts also on eggs deprived of their jelly coats. Wicklund (13) mentioned already briefly that even naked eggs are sensitive to FeInh[Fu]. Table II shows that FeInh[Fu] causes a strong inhibition also in eggs deprived of their jelly coat. However, they are somewhat less sensitive to the inhibitor than the eggs surrounded by a jelly coat. This means that the jelly coat plays a certain role in preventing the fertilization in the presence of FeInh[Fu]. Obviously the penetration of the spermatozoa is somewhat hampered due to the condensed state of the jelly in presence of the inhibitor. On the other hand, it is obvious that the inhibitor exerts a pronounced effect also on the cytoplasmic surface of the egg.

A comparison of Figs. 5 and 6 is of some interest. They represent eggs deprived of their jelly coat. In the one case (Fig. 5) the fertilization occurred in normal sea water. A slightly folded membrane was rather well elevated. Fig. 6, on the other hand, illustrates the behaviour of the eggs in presence of low concentration 0.002–0.004 per cent FeInh[Fu] SupC_4I. The membrane elevation was more irregular. The strong contraction of the egg surface was particularly conspicuous. Mostly two indentations appeared on the egg surface, one somewhat deeper than the other. Above these indentations the membrane was more strongly elevated. The perivitelline space contained a number of particles, granular or rod-shaped. Particles were much more uncommon in the perivitelline space of the control eggs (Fig. 5).

Moreover experiments were carried out on eggs deprived of their jelly coat to decide whether periodate pretreatment renders these eggs less sensitive to the inhibitor, cf. previous section. There seemed to be a shift in the same direction as in the experiment on eggs with the jelly coat intact (Table I). Of four experiments however one contradicted this conclusion. Therefore

[1] Miss Karin Sundius has devoted a more detailed study to the radialising effect of FeInh[Fu]. The threshold concentration of FeInh[Fu] may vary in different batches of eggs.

TABLE II

Paracentrotus lividus, Naples, 20.II.1954. The eggs of one female were divided into two parts. In one the eggs were used with intact jelly coat (I). In the second (II) the jelly coat was removed by brief treatment ~ 1 minute with sea water rendered acid (pH ~ 4.5) by addition of 1/10 N HCl. After the treatment the eggs were thoroughly washed with pure sea water and added to the different media. After 3 minutes the eggs were inseminated.

The examination of the eggs was made 40–55 minutes after the insemination. The percentage figures are based on counts of 100–130 eggs in each case.

Concentration of FeInh[Fu] $SupC_4I$	I. Eggs with jelly coat	II. Eggs deprived of their jelly coat
a. 0 per cent	100 per cent fertilization. Membrane rather low.	100 per cent fertilization, membrane well elevated but folded and easily breakable.
b. 0.009 per cent	100 per cent fertilization, membrane lower than in I, *a.*	100 per cent fertilization, membrane smoother and more solid than in II, *a.*
c. 0.004 per cent	7 per cent of the eggs fertilized.	90 per cent fertilization, membrane low and of very irregular shape.
d. 0.007 per cent	0 per cent fertilization.	15 per cent fertilization. Membrane when present adhering to the egg surface.
e. 0.010 per cent	0 per cent fertilization.	2 per cent fertilization.

the question cannot be regarded as definitely settled. The presence of the jelly coat tends to render the difference between the pretreated and the non pretreated eggs more conspicuous.

Fertilization inhibitor from Fucus and polyspermy

According to Runnström and Kriszat (9) there is a tendency to polyspermy when the eggs are fertilized in presence of dissolved jelly coat substance. The same seems to hold for solutions of FeInh[Fu] in sea water. A high incidence of polyspermy was observed in an experiment recorded in Table III. The eggs had been pretreated with $4 \cdot 10^{-5}$ M periodate and then kept in sea water for 3 ½ hours. The inhibitory effect of FeInh[Fu] was decreased by this treatment, but all the fertilized eggs became polyspermic. When the FeInh[Fu] was further diluted the percentage of polyspermic eggs decreased rapidly.

TABLE III

Paracentrotus lividus, Naples 25.IV.1953. Eggs in sea water or in sea water with FeInh[Fu] (preparation Sup C_4 I) were fertilized with two different sperm suspensions: prepared by adding one drop "dry sperm" to 10 ml or to 2 ml sea water. Insemination was carried out by adding one drop of the weaker or the stronger sperm suspension to 1 ml egg suspension 3–4 minutes after the transfer of the eggs to the respective media. The following observations were made 90 minutes after insemination.

Medium	Weaker sperm suspension	Stronger sperm suspension
a Sea vater	95 per cent regular 2-cell stage. 5 per cent polyspermic eggs.	72 per cent regular 2-cell stage. 28 per cent polyspermic eggs.
b. Sea water with 0.006 per cent FeInh/Fu/	86 per cent unfertilized eggs. 14 per cent uncleaved polyspermic eggs with no membrane elevation.	20 per cent unfertilized eggs. 80 per cent uncleaved polyspermic eggs with no membrane elevation.

One more feature of this experiment should be commented upon. The number of fertilized eggs in presence of FeInh[Fu] increases considerably with the number of spermatozoa. This has been confirmed in several experiments. There seems thus to be an antagonism between some factor carried by the sperm and FeInh[Fu]. It is difficult to say if this factor resides in the spermatozoon itself or in the sperm liquor. Hultin *et al.* (4) centrifuged heavy suspensions of *Psammechinus* eggs, resuspended them and centrifuged again. In this manner several supernatants were obtained. The first supernatants contained a factor which causes a strong swelling of the jelly coat and improves the membrane elevation. FeInh[Fu] inhibits the swelling action of these supernatants. The effect depends on the balance between the antagonizing factors so that in sufficient concentration the sperm supernatant may counteract the condensing and even the fertilization inhibitory effect of FeInh [Fu].

In some experiments it was shown that addition of periodate to the FeInh[Fu]-solution in sea water removes the polyspermy enhancing effect of FeInh[Fu]. In one experiment for example, two test media were prepared, one containing 0.01 per cent FeInh[Fu] (I) and a second with the same concentration of FeInh[Fu] but with the addition of $2 \cdot 10^{-5}$ M sodium periodate (II). The eggs inseminated in I contracted very strongly and formed a very low thin membrane. The incidence of polyspermy was high. In II, on the other hand, a membrane elevated without strong contraction of the egg. No polyspermy was observed. The larvae that developed in I were radially symmetrical, whereas in II bilateral plutei appeared.

DISCUSSION

The data submitted above contribute to elucidating the inhibitory effect exerted by the factor contained in the extract of *Fucus vesiculosus*, the fertilization inhibitor from Fucus (FeInh[Fu]). This prevents the swelling of the jelly

coat which normally occurs when the eggs are kept in pure sea water. It was of particular interest to observe the inhibitory effect on the action exerted by the sperm on the jelly coat (p. 3). The swelling and dissolving effect of the sperm on the jelly coat or fragments of this was blocked in presence of FeInh[Fu]. The inhibitor seems also to influence the jelly substance in solution. This follows from the delay in dissolution of the loose aggregations that form after the initial head agglutination, at least under the conditions of the observations reported above. This points to the conclusion that the spermatozoa when agglutinating gather around invisible fragments of jelly. On a smaller scale the same phenomena are repeated as those observed when spermatozoa attack larger fragments of the jelly. A swelling occurs which dislocates the spermatozoa so as to remove them a certain distance from each other. Under normal conditions the swelling results in a dispersion of the jelly material. In presence of the FeInh[Fu] this final dispersion of the jelly material is delayed or fails to occur. It is obvious that the FeInh[Fu] strongly affects the water binding capacity of the jelly coat. The assumption of a cross linkage of fibre or sheetlike structural elements would account for the changed conditions in the jelly. The cross links can evidently not or only with difficulty be broken up by the spermatozoa.

If now the attention is turned to the cytoplasmic surface of the egg striking analogies are found to what has been reported for the jelly coat. Even if the jelly is removed a sufficient concentration of FeInh[Fu] will suppress the activating effect of the spermatozoon. The threshold concentration may be somewhat higher when the eggs are deprived of their jelly coat (p. 8, 9) but the role of the jelly for the inhibitory effect is comparatively unimportant. Again the concept of a cross linking of structural elements in the egg surface seems to account for the abnormal conditions of the cytoplasmic surface. This concept receives support from independent evidence, *viz.* by the results of Kriszat (5) obtained with the help of the centrifuge microscope. He followed the elongation of jelly-free eggs suspended on the border between sea water and isotonic cane sugar solution. The rate of elongation in the control was considerably greater than in test media with 0.05 per cent FeInh[Fu] B 6. On the other hand pretreatment with $5 \cdot 10^{-5}$ sodium periodate enhanced the rate of elongation quite considerably. These results would in themselves suggest the same explanation as the one proposed for the action of FeInh[Fu] on the jelly. FeInh[Fu] increases the cross linkages in the cytoplasmic surface, thus increasing its rigidity. Sodium periodate on the other hand decreases the linkages between the elementary units of the cortical layer. In one experiment Kriszat (5) subjected the egg for 30 minutes to 0.05 per cent

FeInh[Fu] B 6. Only thereafter the eggs were transferred to $6 \cdot 10^{-5}$ periodate in sea water. The decrease in rigidity of the egg surface was lesser in these eggs than in those which were directly transferred to periodate solution. The FeInh[Fu] thus reduces the sensitivity to periodate. On the other hand it has been shown above that periodate renders the eggs less sensitive to FeInh[Fu]. This is indicated by the higher concentrations of FeInh[Fu] needed to bring about inhibition of fertilization after pretreatment with periodate (p. 5). These results are in keeping with the view that there is a natural inhibitor present in the egg surface. The treatment of the egg for example with periodate decreases the action of the natural inhibitor and thus improves fertilization, *cf.* also (8). The effect of FeInh[Fu] seems to be additive to the effect of the natural inhibitor. Thus the egg is brought down on the scale toward greater refractoriness. Concentrations of FeInh[Fu] which in themselves cause no block of fertilization bring about a strong contraction upon fertilization, see above Fig. 6. As pointed out by Wicklund (13) the conditions are reminiscent of those prevailing in underripe eggs.

Harding (3) already demonstrated that heparin blocked the fertilization or at least the normal membrane formation even after removal of the jelly coat. One preparation of heparin was found to be especially active. The heparin must perhaps have a special configuration or a related more active substance was present in the preparation in question. The autoclaving of less potent preparations increased their inhibitory action (2). The local contractions conspicuous in eggs fertilized in presence of FeInh[Fu] were found in experiments with heparin. The action of both substances is certainly very much related. It is remarkable that their effect is easily reversible upon washing of the eggs with sea water (2, 3).

From the data in Table III it was inferred that the sperm contains factors counteracting the effect of FeInh[Fu]. The mutual inhibitory effects of sperm factors and FeInh[Fu] depend obviously on relative concentrations and other circumstances which influence the activity of the respective factors. The sperm factors in question may also be operating in the elimination of the natural inhibitor of the egg surface. As pointed out above, see also (4), the sperm contains factors which bring about a swelling or even dissolution of the jelly coats.

It has been mentioned above that the presence of FeInh[Fu] induces a development of radially symmetrical larvae. In this respect the results are similar to those obtained by Gustafson and Sävhagen (1) with anionic detergents. Also the sensitive periods are the same. It is of interest that the fractions of the *Fucus* extract most potent as fertilization inhibitors were also the most

efficient in inducing a radially symmetrical organisation of the larvae. A common underlying factor is probably operating in both cases. It may be of interest to mention that also heparin has a pronounced radializing effect on the development. This was found in unpublished work by Gustafson for *Echinocardium cordatum* and by Runnström for *Arbacia lixula.*

SUMMARY OF RESULTS

The purpose of this paper was to contribute to the analysis of the inhibitory effect exerted by an extract from *Fucus vesiculosus* on fertilization of the sea urchin egg. The active agent has been purified to a certain extent by Vasseur *et al.* (12). The agent is designated "Fertilization Inhibitor from *Fucus*" (FeInh [Fu]).

FeInh[Fu] inhibits the swelling of the jelly coat surrounding the sea urchin egg (*Arbacia lixula, Paracentrotus lividus, Psammechinus miliaris*).

Sperm of high concentration caused a swelling and dissolution of the jelly coat of eggs of *Arbacia lixula.* This effect is counteracted by FeInh[Fu].

The agglutination of the spermatozoa of *Paracentrotus lividus* and *Arbacia lixula* showed under certain defined conditions two different phases, a longer initial phase of head agglutination and a transient phase of loose aggregation. The second phase was stabilized in presence of FeInh[Fu]. The results are interpreted as being due to a decreased depolymerization of the dispersed particles of the jelly coat substance in presence of the inhibitor.

Pretreatment of the eggs of *Paracentrotus lividus* with sodium periodate ($4 \cdot 10^{-5}$ M) rendered the eggs less sensitive to FeInh[Fu].

This statement applies in the first place to eggs surrounded by their jelly coat. Incomplete evidence indicated that it holds likewise for naked eggs.

Evidence is given to show that also naked eggs are susceptible to the inhibitory action of FeInh[Fu], see (13). The results indicate, however, that the condensed state of the jelly coat in presence of FeInh[Fu] contributes to some extent to the inhibition of fertilization.

In concentrations of FeInh[Fu] which allow fertilization of a number of the eggs a high incidence of polyspermy was observed (*Paracentrotus lividus*).

Increase in the amount of sperm is apt to break down the resistance offered by FeInh[Fu] against fertilization, see Table III. Delayed fertilization of eggs subjected to FeInh[Fu] indicates also that the resistance offered by the inhibitor is not an absolute one but it can be overcome by factors acting in the sperm.

Brief mention is made of the fact that eggs subjected to low concentrations

of FeInh[Fu] develop into radially symmetrical larvae. Heparin acts in the same way. Periods of sensitivity etc. are similar to those observed for the action of detergents, see (1).

The results are interpreted by assuming that FeInh[Fu] causes an increased cross linkage in the jelly substance and in the cortical layer of the cytoplasm as well.

The action of FeInh[Fu] is synergic with that of a natural inhibitor present in the cytoplasmic surface of the egg. It is less accessible to the attack of the sperm factors than the natural inhibitor but, as stated above, the resistance is not an absolute one.

The experimental work was mainly carried out at Stazione Zoologica, Naples. We wish to express our sincere thanks to Professor Reinhard Dohrn for his kind interest and generous help. Supplementary work was carried out at the Swedish Zoological Station at Kristineberg. We are obliged to Dr. G. Gustafsson for his unfailing helpfulness. Finally we express our thanks to Dr. Erik Vasseur and Miss Ulla Björklund for generous supply of the fertilization inhibitor from *Fucus*. The work was supported by a grant from the Swedish Natural Sciences Research Council.

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THE ULTRASTRUCTURE OF CHLOROPLASTS

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The chloroplasts of higher plants consist of the grana, which contain the pigments, and of a colorless stroma. The grana are easily observed with the light microscope (6) as well as with the electron microscope (1, 5). As they contain the pigments of the leaves they are attributed special importance in connection with the process of assimilation.

Some observations have already indicated that the grana have a layered structure:

a) Through a careful isolation of chloroplasts from *Aspidistra* it has been shown by means of electron microscopy that the grana consist of piles of thin discs, which turn over when dried on a supporting film (11).

b) When chloroplasts that have been fixed are subjected to sonic treatment, circular discs are obtained. The diameter of these discs corresponds to the diameter of the grana and their thickness is about 70 Å (12).

c) On thin sections through chloroplasts a regular organization of the grana has been observed (2, 7, 8, 12).

The limitations of the generally applied techniques for preparing ultrathin sections have until now made it impossible to draw any definite conclusions regarding the ultrastructure of the grana as to their form, arrangement, and mutual connections. Neither the organization of the stroma has been accessible for an ultrastructural analysis.

MATERIAL AND METHODS

The study was performed on *Aspidistra elatior*. For fixation osmium tetroxide was used as representing the best fixative for lipoprotein structures.

The preparatory work was performed in a cold room at 0° C. Leaves of *Aspidistra* were cut in about 0.1 mm thick slices by means of a razor blade knife. The cut surface was all the time in contact with the osmium tetroxide solution. The thin slices were fixed for about 5 hours in 1 per cent osmium tetroxide solution at 0° C. The osmium tetroxide was dissolved either in a veronal acetate buffer of pH 6.9–7.2 the molar concentration of which was about 0.32, or in a 0.25 M solution of sucrose. After fixation the slices were fragmented by means of a Waring blendor. The cell walls

were removed through centrifugation the supernatant representing a dispersion of chloroplasts. After washing the chloroplasts were dehydrated and embedded in methacrylate.

The ultrathin sections were prepared using the type of ultramicrotome designed by Sjöstrand (9). They were examined in an RCA EMU 2 c electron microscope without dissolving or subliming the methacrylate.

RESULTS

The arrangement and the form of the grana. The grana are easily recognized in ultrathin sections due to their very regularly lamellated structure (Fig. 1, 2). As could be expected from earlier electron microscopic studies the grana are not lense-shaped, that is the form they frequently show when examined in the light microscope. They rather represent long columns with the diameter varying along its course (Fig. 1). A more or less elliptic outline is observed only when the grana are cut obliquely (Fig. 5). The length of the grana columns is not constant. As an extreme such a column may extend all through the chloroplast. Generally the grana columns consist of 20–30 opaque layers. This figure may, however, exceed 100 (Fig. 1). As is seen in Figs. 1, 2, and 6 two adjacent grana may be mutually connected through a series of layers extending through both grana.

The significance of the very opaque spherical granules observed in the stroma is obscure. They appear in groups rather randomly distributed over the chloroplast stroma and their diameter measures up till 0.18 μ. They might represent reserve material or might be the result of a redistribution of the lipid molecules during the fixation. They may be observed in fragmented chloroplast material and, therefore are not formed as an effect of the embedding.

The lamellated structure of the grana. The new technique for ultrathin sectioning makes it possible to confirm the lamellated structure of the grana in a rather beautiful way. The lamellation is most obvious when the lamellae are cut perpendicularly. The opaque layers alternate with less opaque interspaces in a strikingly uniform way. When cut obliquely the layers appear thicker than they really are. Therefore, when considering the values obtained when measuring the dimensions the minimum values should be considered the most reliable. The thickness of the alternating opaque and transparent layers was measured in regions where the layers appeared most regularly arranged and were found to be equally thick and measuring about 65 Å. The opaque layers represent membranes or discs.

When looking more closely at the layered structure it appears that two

opaque layers are connected at their rim forming pairs of discs. (Figs. 4, 6.) The grana therefore seem to consist of double membrane discs in which a closed narrow space exists the height of which is about 65 Å. The distance from the center of such a fundamental unit to the next is 250–270 Å (Fig. 7). The distance between the adjacent surfaces of two double membrane discs is about 65 Å, that is the same distance as between the two opaque layers within the disc.

The pronounced electron scattering effect of the opaque layers certainly is due to an electron staining through the osmium tetroxide. Unfortunately, there is little background for conclusions regarding the chemical composition of the opaque and transparent layers within the grana, the chemical background for the osmium staining not being well understood.

The connections between the grana. As the grana are embedded in the chlorophyll-free stroma it is of interest to know whether they are mutually connected and thus kept oriented in defined positions or are freely spread out in the stroma. Strugger (13) assumed from experiments on the swelling of the grana that they were mutually connected through so called "Trägerlamellen".

In ultrathin sections it is shown that the stroma exhibits a layered structure (Figs. 3, 5). The opaque layers of the stroma are, however, much thinner than those within the grana. Their thickness measures about 30 Å, a dimension close to the resolution of the EMG:s on which this analysis was based. From each opaque disc of the grana one such thin membrane extends across the stroma (Figs. 3, 6, 7).

The membrane structure of the stroma seems to be more susceptible to the preparative treatment than the grana discs. Already minute changes of the osmotic conditions may produce a swelling of the stroma with a separation of the grana.

The chloroplasts of higher plants thus are organized in continuous layers as has earlier been demonstrated for chloroplasts from algae (11) and from flagellates (13, 14). The lamellae of the stroma are, however, thin and labile as compared to the stable regularly lamellar structure of the grana. This continuity of the layering all through the chloroplasts might well represent a universal scheme according to which all chloroplasts are organized.

The swelling of the chloroplasts from Aspidistra. When immersed in distilled water the chloroplasts from *Aspidistra* form long, frequently branched threads. These threads consist of spherical vesicles lined up like a beaded string. These threads contain chlorophyll and show the diameter of the grana (4).

This striking effect of swelling is easily explained when considering the organization of the grana in units of double membrane discs. The effect would be expected if these double layered discs consist of semipermeable membranes or the material in between these structural units has the tendency to swell. This susceptibility to hypotonic media seems to be one reason for the distortion following improper fixation.

DISCUSSION

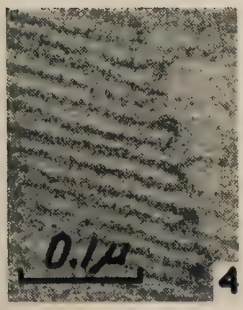
With the highly improved techniques for preparing ultrathin sections the ultrastructure of the chloroplasts may now be directly visualized. The earlier studies by Wolken and Palade (14, 15), Leyon (7, 8) and by Cohen and Bowler (2) have proved the layered structure of the chloroplasts. It may be assumed that the ultrastructure of the chloroplast varies to some extent in different species of plants. This would account for the great divergency of our observations from those made by Cohen and Bowler (2) on chloroplasts from tobacco plants. Their pictures show a regular linear arrangement of individual grana in complete accordance to the "Geldrollen" assumed by Strugger (13). The irregularities regarding the mutual relationships and the diameter of the grana in *Aspidistra* might perhaps be correlated to this plant representing an extreme representative of a plant growing in the shadow. A study of chloroplasts from other species is necessary to prove this assumption.

The ultrastructure of the grana from *Aspidistra* shows a striking similarity to that of the outer segments of the retinal rods of the vertebrate retina (10). The grana as well as the rod outer segments consist of double membrane discs. The similarity might be due to both elements being responsible for a transformation of light energy into chemical energy, a reaction that obviously is confined to lipoproteins organized in layered structures.

The retinal rod outer segments differ structurally from the chloroplasts, the double membrane discs in the former constituting a continuous structure through the whole mass of the outer segment as compared to the limited regions of the chloroplasts which are organized as grana. From this point of view it would be expected to obtain better defined low angle x-ray scattering from fixed retinal rod outer segments than from *Aspidistra* chloroplasts as was also found in an earlier study (3). The repeating unit then calculated from the x-ray diffraction patterns was 250 Å which confirms in a strikingly exact way the measurements performed on the ultrathin sections.

Fig. 1. Chloroplast from *Aspidistra elatior*. Survey picture. Magnification: 43,000 ×.

Fig. 2. Part of Fig. 1 at a higher magnification. Two adjacent grana are connected through grana membranes passing through both grana. Magnification: 116,000 ×.



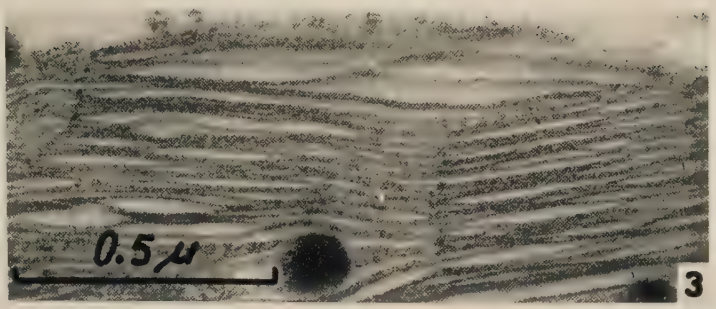


Fig. 3. Chloroplast from *Aspidistra elatior*. The lamellae of the stroma are clearly seen in between the grana. Magnification: 68,000 ×.
Fig. 4. Chloroplast from *Aspidistra elatior*. High magnification of the peripheral part of a grana showing the grana membranes connected in pairs. Magnification: 157,000 ×.
Fig. 5. Chloroplast from *Aspidistra elatior* with several grana sectioned obliquely to the plane of the grana membranes. Magnification: 62,000 ×.

Fig. 6. Chloroplast from *Aspidistra elatior*. High magnification picture of grana. Magnification: 160,000 ×.

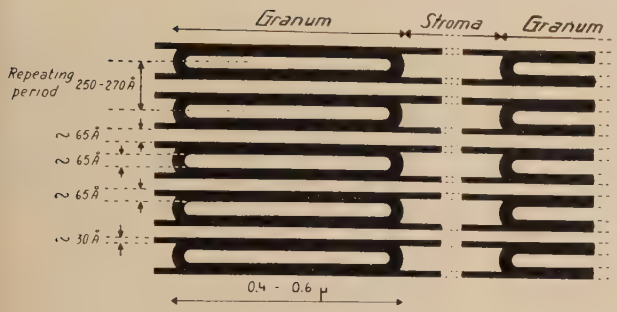


Fig. 7. Schematic presentation of the organization of the chloroplast (*Aspidistra elatior*) grana and stroma. The diameter of the grana and the distance between the grana are not drawn according to scale.

SUMMARY

1. On ultrathin sections of chloroplasts from *Aspidistra elatior* the electron microscopic study has revealed that the grana consist of layered columns.

2. The grana are organized in double membrane discs the two membranes of which are about 65 Å thick. These membranes bound a narrow, closed space the height of which also is about 65 Å. The spacing of this double membrane structure was measured to 250–270 Å. The double membrane discs easily swell when subjected to hypotonic media.

3. The double membrane discs of adjacent grana are mutually connected through about 30 Å thick membranes extending through the stroma. This structure of the stroma shows obvious tendencies to swell in hypotonic media.

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THE EFFECT OF ANIONS ON THE OPTICAL PROPERTIES OF RAT LIVER NUCLEI ISOLATED IN GLYCEROL SOLUTIONS[1]

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Although nuclei isolated in bulk quantity from rat liver tissue have been the subject of considerable biochemical investigation during the past decade, the importance of establishing the cytological integrity of such nuclei has received attention only recently. The original emphasis upon recovery and purity of nuclear preparations as primary requisites for their biochemical analysis was accompanied by a general indifference to microscopic alterations imposed upon nuclei by the isolation procedure employed.

The demonstration, by several investigators (13, 15, 19), that nuclei freed from tissue fragments in sucrose were optically similar to those of intact cells, was succeeded by the isolation of optically clear rat liver nuclei in quantities sufficient for biochemical analysis (22). Although the nuclei prepared from buffered sucrose were clustered and contaminated with mitochondria, the work of Wilbur and Anderson (22) introduced a new emphasis upon thorough microscopic examination of cytological detail as a criterion for nuclear preparations. This emphasis upon simulating the optical properties of intracellular nuclei led, more recently, to the isolation of optically homogeneous liver nuclei from sucrose solutions containing calcium chloride (11), in the salt concentration previously selected for the preparation of spleen nuclei (18).

Despite the cytological superiority of nuclei obtained in both types of sucrose media mentioned above, the suspected loss of water-soluble components from nuclei isolated in aqueous solutions (14, 6, 18 a) has promoted the use of more drastic fractionation methods involving organic solvents (8, 2, 12, 20). It therefore seemed desirable to investigate the possibility of replacing sucrose with some nonelectrolyte which would permit the isolation of nuclei in a medium containing a minimum of water, without forfeiting the relatively simple techniques employed with sucrose solutions.

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The present work describes the use of 70 per cent glycerol in a rapid and simple procedure for the isolation of rat liver nuclei. The method was applied to a study of the effects of various reagents upon the cytology of these nuclei, examined under dark phase contrast and dark-field illumination. The results are in agreement with those previously reported by Anderson and Wilbur (3) on the microscopic changes induced by the addition of electrolytes to suspensions of nuclei isolated in buffered sucrose. The glycerol method, however, differs in approach by permitting the incorporation of test solutions in the suspending medium as part of the procedure for isolating the nuclei in a variety of optical states, as desired. This approach has the advantage of making possible a study of factors concerned with nuclear dispersion and clustering, in addition to those concerned with optical homogeneity.

METHODS

Criterion of "living" cell nuclei. The criterion adopted for the natural state of interphase nuclei was the microscopic appearance of hepatic nuclei released from fragments of rat liver crushed in the host's plasma. This criterion was adopted arbitrarily, notwithstanding the two major objections that a nucleus dislodged from its original position in an integrated cell is automatically divested of its natural state (4) and that an extracellular medium is not necessarily a natural environment for an intracellular component (3).

For the preparation of plasma, the rat was anesthetized ether and 5 ml of cardiac blood drawn into a syringe rinsed with heparin. The liver of the same animal was then excised immediately, and a tiny fragment of the tissue was removed from microscopic examination. The fragment was freed of a large proportion of erythrocytes and cytoplasmic debris adhering to the excised tissue, by being dipped through nine successive single drops of plasma on a slide before being finally minced in the tenth drop. The hepatic nuclei floating freely in the last drop were sufficiently dispersed to permit detailed microscopic examination.

Suspending solution. Seventy per cent glycerol was used as the suspending medium throughout this work. For a study of test reagents, the glycerol solution was prepared to contain the desired concentration of the specific reagent. In order to ensure proper mixing, the solution of test reagent was diluted with water prior to the addition of the necessary volume of concentrated glycerol for adjustment to 70 per cent.

Wherever possible, the inorganic salts used were selected as potassium salts. The organic reagents sequestrene and versene were sodium salts of polyaminocarboxylic acids.

Isolation procedure. The procedure described below represents an adaptation of the method previously devised by the author for the isolation of spleen nuclei in sucrose (18). Rats of the Long and Evans strain were sacrificed by a head blow and partially exsanguinated by permitting the blood to drain from the nostrils. The livers were excised, cut into small cubes, and immediately frozen on dry ice. The cubes

of tissue were stored at −20° C and used, as desired, up to a maximum period of 48 hours, following which they were discarded.

A one-gram mass of tissue was suspended in 15 ml of the desired medium and crushed in a ground glass homogenizer, inside diameter one inch, by means of 20 up and down strokes of the tube against a stationary pestle. Preliminary clarification was effected by straining the homogenate through a double layer of surgical gauze, followed by filtration through single-napped flanelette, into a 50-ml glass centrifuge tube. Since microscopic examination at this stage showed the presence of frequent sheets of whole cells, the final filtrate was dispersed, for five minutes, in the centrifuge tube, by means of a notched stainless steel blade attached to the shaft of a motor rated at 22,000 r.p.m. During this high speed rotation, the centrifuge tube containing the homogenate was kept in a beaker of crushed ice, properly packed to maintain a temperature of 2° C.

Effective separation of the nuclei from the homogenate was accomplished by the use of the "layering" technique (10, 22, 1, 11). The homogenate was simply poured down the side of a 15-ml plastic tube containing one ml of the suspending medium, where it remained as a turbid red phase overlying the one ml of clear solution. The contents of the plastic tube were then centrifuged for ten minues at 2,700 × g in an International cold centrifuge, model PR-1, maintained at O° C. Under these conditions, a large number of nuclei traversed the interface and accumulated at the bottom of the clear solution, whereas the lighter components remained in the overlying red homogenate. The supernate was discarded, and the nuclear sediment was washed twice by centrifuging at 2,700 × g, at 10 minutes intervals, in 10 ml portions of the original medium. The final sediment was suspended in one ml of solution, was stirred with a fine glass rod, and was then ready for microscopic examination.

Microscopic examination. All samples were examined as unstained wet mounts, prepared by applying to a slide a small drop of suspension from the end of a fine glass rod and placing a cover glass over the drop. Darkfield illumination, at a magnification of 1125 ×, and dark phase contrast, at a magnification of 1213 ×, were used routinely for examination of nuclear detail.

RESULTS

Nuclear morphology

The hepatic nuclei suspended among the debris of the plasma homogenate showed features typical of these nuclei; i.e., spherical form, opaque nucleoli, and smooth nuclear membranes. The nuclei otherwise were not entirely optically clear under phase contrast, but showed evidence of a very faint opacity, suggestive of an extremely fine granularity. The slight extent of this opacity is best indicated by the fact that it was insufficient to cause light refraction under darkfield illumination.

The use of glycerol solutions permitted the isolation of hepatic nuclei showing three different types of nuclear texture. These types have been

designated as granular, homogeneous, and hyaline, and may be described briefly as follows:

"*Granular*" *nuclei*, as represented in Fig. 1, were characterized by optical heterogeneity, manifested as an evenly distributed coarse granularity. Under darkfield illumination, the coarse areas showed up as brightly refracting light points against a dark background. The nuclei were spherical and dispersed, and the nuclear membranes remained smooth and intact. The nucleoli were observed to consist of several opaque segments, folded close together to form compact structures. These nucleolar segments were also recognizable under darkfield illumination by their distinctive refractile pattern.

"*Homogeneous*" *nuclei*, as represented in Fig. 2, were characterized by a relatively structureless appearance, with only the faintest optical opacity, if any. The size, state of dispersion, and nucleolar structure of these nuclei were similar to those of the granular nuclei. Under darkfield illumination, these nuclei appeared as round black spheres, refracting light only at the segmented nucleoli and at the nuclear surface. The appearance of this type of nuclei was very similar to that of the nuclei seen in plasma homogenates.

"*Hyaline*" *nuclei*, as represented in Fig. 3, were characterized by a completely structureless appearance, devoid of opacity and nucleoli, and presenting the appearance of transparent droplets. The nuclei were obviously enlarged and invariably aggregated into clusters which resisted all attempts at dispersion. Under darkfield illumination, this hyaline effect was confirmed by the absence of refracting points except at the nuclear surface.

Microscopic effects of test reagents

Dispersed nuclei: The nuclei isolated from solutions of 70 per cent glycerol alone were dispersed and extremely coarse in texture, resembling nuclei isolated in citric acid (7). Neutralization of the glycerol medium with potassium hydroxide resulted in a conspicuous diminution of this coarseness, but a residual granularity persisted, as shown in Fig. 1.

In view of the recent use of buffer salts in the isolation of optically clear nuclei (22), it seemed advisable to note the effect of replacing the potassium hydroxide with buffer salts. The acknowledged granulation effect of divalent cations (3) suggested the possibility that the induction of optical homogeneity by buffer salts might be related to the metal-combining ability of their anions. Various metal-binding substances, in the form of both inorganic electrolytes and organic chelating agents, were incorporated in the 70 per cent glycerol,

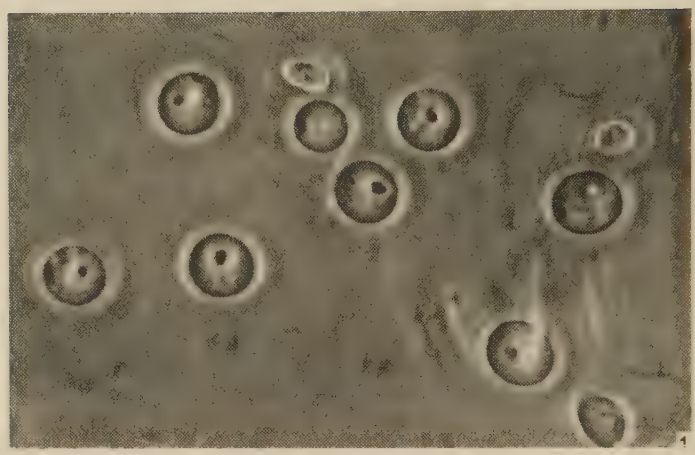

Fig. 1. Granular, dispersed nuclei isolated in 70 per cent glycerol containing 0.0002 M potassium hydroxide. Dark phase contrast. 1200 ×.

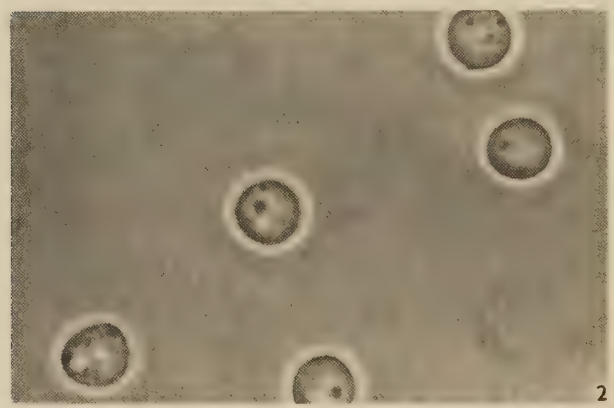

Fig. 2. Homogeneous, dispersed nuclei isolated in 70 per cent glycerol containing 0.002 M potassium dihydrogen phosphate and 0.002 M dipotassium hydrogen phosphate. Dark phase contrast. 1200 ×.

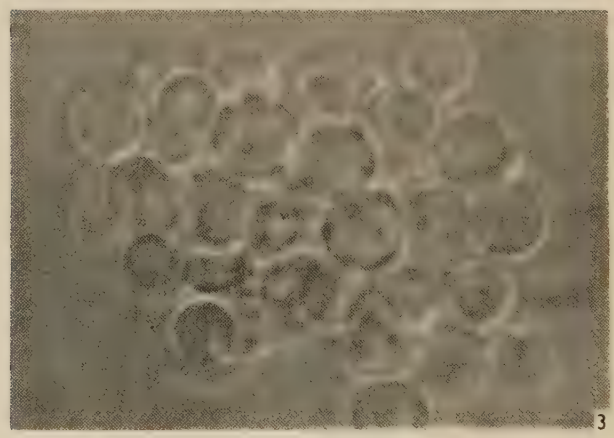

Fig. 3. Hyaline clustered nuclei isolated in 70 per cent glycerol containing 0.0005 M potassium citrate. Dark phase contrast. 1200 ×.

TABLE I

Reagents yielding optically homogeneous nuclei in 70 per cent glycerol.

Test Reagent	Granular appearance maintained at		Nongranular appearance visible at	
	M conc.	pH	M conc.	pH
$(NaPO_3)_6$	0.00024	6.6	0.00032	6.7
$K_3C_6H_5O_7$	0.0002	6.8	0.0004	7.2
KOH	0.0008	8.9	0.001	9.6
K_2CO_3	0.0008	9.4	0.001	9.6
K_3PO_4	0.0008	9.4	0.001	9.7
K_2HPO_4	0.0007	7.6	0.001	7.7
Sequestrene-Na2	0.0039	6.5	0.0039	6.7
+ KOH	0.0005		0.001	
Versene	0.00095	8.3	0.0010	8.6
KPO_3	0.0014	7.6	0.0015	7.7
K_2HPO_4	0.0018	7.2	0.0020	7.2
KH_2PO_4	0.0018		0.0020	
$K_2C_2O_4$	0.002	6.3	0.004	6.5
KCl	0.030	5.5	–	
KNO_3	0.030	5.8	–	
$KC_2H_3O_2$	0.030	5.7	–	

and the textures of the nuclei isolated from these media were compared. Nuclei isolated from glycerol solutions containing such reagents showed a progressive decrease in granular appearance as the concentration of the reagent was increased.

Table I lists the concentrations of the reagents tested and the pH values at which optical homogeneity (Fig. 2) was first clearly apparent. Since the intervals of concentration necessarily varied in accordance with the effectiveness of each reagent, the tabulation also includes the values at which granularity was still clearly visible.

Of the 14 reagents tested, the 11 which were effective in yielding nongranular nuclei all contain anions which have the ability to combine with polyvalent cations. The control anions nitrate, chloride and acetate, were ineffective, even at high concentrations, and yielded only granular nuclei. The wide range of pH values for the media yielding optically homogeneous nuclei tends to minimize the role of neutrality *per se* in this phenomenon.

TABLE II

Comparison of concentrations of reagents in 70 per cent glycerol yielding dispersed and clustered nuclei.

Test Reagent	Dispersed, Homogeneous		Clustered, Granular		Clustered, Hyaline	
	M conc.	pH	M conc.	pH	M conc.	pH
$(NaPO_3)_6$	0.00032	6.7			0.0004	7.1
$K_3C_6H_5O_7$	0.0004	7.2			0.0005	7.3
KOH			0.0008	8.9	0.0010	9.6
K_2CO_3			0.0008	7.6	0.0010	8.3
Versene	0.0010	8.4			0.0011	8.5
KPO_3	0.0015	7.7			0.0016	7.8
K_2HPO_4	0.001	7.7			0.002	7.9
K_3PO_4	0.001	9.7			0.002	9.7
$K_2C_2O_4$	0.004	6.5			0.008	6.5

Clustered nuclei. Clustering of nuclei is a difficulty commonly experienced by investigators engaged in the preparation of nuclear fractions (22). This condition of nuclear aggregation is undesirable because it invariably causes trapping of contaminants within the aggregates and, moreover, prevents numerical counting of the nuclei. The phenomenon of nuclear clustering, therefore, warranted investigation.

The hyaline appearance of the clustered nuclei obtained in buffered sucrose by Wilbur and Anderson (22) suggested to the writer the possibility that clustering might be associated with hyalinity. It was also suspected that a concentration of salt exceeding that yielding dispersed homogeneous nuclei might produce the extreme condition of hyalinity, accompanied by nuclear enlargement and clustering. Therefore, several of the reagents were tested, in progressively increasing concentrations, until the nuclei isolated from the glycerol-reagent solutions appeared clearly clustered.

Table II lists the concentrations of the reagents selected and the pH values at which the nuclei first appeared clustered. For the sake of comparison, the data for the dispersed homogeneous nuclei are also included. The data indicate that in the majority of instances, the concentration of reagent required for nuclear clustering did exceed that causing nuclear dispersion with granularity. The two exceptions where clustered nuclei retained a granular appearance involved the hydroxyl and carbonate ions.

TABLE III

Comparison of salt concentrations required in aqueous and in glycerol solutions for induction of optical homogeneity.

Test Reagent	M Aqueous Solution	M Glycerol Solution	Aqueous/ Glycerol
$(NaPO_3)_6$	0.00006	0.00032	0.20
$K_3C_6H_5O_7$	0.0001	0.0004	0.25
K_2CO_3	0.0002	0.0010	0.20
KPO_3	0.0005	0.0015	0.33
$K_2C_2O_4$	0.001	0.004	0.25
			Mean 0.25

Reversibility of optical states

For a study of the reversibility of optical states of the isolated nuclei, preparations of dispersed nuclei only were used. A drop of the aqueous test reagent was added to a similar volume of nuclear suspension on a slide, the drops were mixed gently with a fine glass rod, and a cover glass was placed over the mixture. The effects of the reagents upon the nuclei were sufficiently rapid to permit immediate microscopic examination.

Granular nuclei, obtained from 70 per cent glycerol neutralized with 0.0002 M potassium hydroxide (Fig. 1), were treated with aqueous solutions of several of the salts previously shown to yield homogeneous (Fig. 2) or hyaline (Fig. 3) nuclei. Each test reagent was added in a progressively increasing concentration until no evidence of granularity remained in the nuclei. Table III lists the concentrations and pH values of the test solutions at which granularity disappeared. The tabulation also includes the data for the same reagents which had previously yielded homogeneous nuclei in glycerol.

Although the concentrations of aqueous salt solutions required to cause optical homogeneity were consistently about 25 per cent of those required by the glycerol-salt solutions, the general trend of effectiveness was the same for both types of solutions. In both cases, the hexametaphosphate salt was the most effective in reversing granularity, and the oxalate salt was least effective.

Optically homogeneous nuclei (Fig. 2), obtained from 70 per cent glycerol containing 0.002 M potassium diphosphate and 0.002 M dipotassium phos-

phate, were treated with divalent ions in the form of chloride salts. At a concentration of 0.1 M, magnesium, manganese and calcium caused an immediate change from homogeneous to granular appearance.

DISCUSSION

The use of glycerol for the isolation procedure described was prompted by the observation that glycerol solutions in high concentrations yielded microscopically intact and dispersed hepatic nuclei, free of extranuclear contaminants (17). The concentration of 70 per cent glycerol finally selected for the suspending medium represents the highest concentration of glycerol which could be manipulated conveniently under the conditions employed. Biochemical studies on nuclei isolated in glycerol solutions of varying densities have since been reported by Dallam and Thomas (5).

Of the three types of nuclei isolated by the method presented, the optically homogeneous type showed the closest resemblance to hepatic nuclei floating in the plasma homogenate. They both lacked the coarseness of granular nuclei and contained opaque nucleoli which were invisible in hyaline nuclei. In addition, the homogeneous nuclei retained the original size and discreteness of intracellular nuclei, in contrast to the hyaline nuclei which were enlarged and clustered. Therefore, it would appear that, of the three nuclear states described, the optically homogeneous condition represents the state of least alteration to hepatic nuclei.

The use of glycerol solutions made possible the isolation of nuclei in a condition morphologically similar to intracellular living nuclei, without the use of sucrose. However, nuclei prepared in 70 per cent glycerol alone showed the features typical of fixed nuclei, including condensed chromatin and nuclear shrinkage. Since both granular and nongranular types of nuclei were obtained from media containing an identical concentration of glycerol, the induction of optical homogeneity in this study cannot be attributed to the presence of a nonelectrolyte (15). It must, instead, be referred to the metal-binding capacity of the anionic radicals of the reagents incorporated in the glycerol solution. Each effective metal-binding anion seemed to operate within a specific concentration range, below which granularity was produced and above which hyalinity was induced. These findings emphasize the role of polyvalent anions in maintaining the structureless appearance of interphase hepatic nuclei.

Although the present work is inadequate to resolve the relationship of structural organization to optical properties of nuclei, certain speculations are

possible. It is of some interest to note that polyvalent cations (3) and dehydrating solvents (23, 15) alike are capable of inducing immediate nuclear granularity. Conversely, cation-binding reagents and distilled water (9, 25) alike are capable of reversing this granularity. These observations tend to confirm the role of hydration (9) in the phenomenon of optical homogeneity and also implicate metallic ions in the phenomenon of dehydration. Some support for these suggestions may be found in X-ray diffraction patterns of brain lipid emulsions (16), which indicate the superior dehydrating effect of electrolytes containing divalent cations, in colloidal systems. One could therefore speculate that the effectiveness of the homogenizing and hyalinizing reagents reported in the present work was proportional to their ability to maintain the hydrated, or extended (15), state of the nuclear structures, by virtue of their ability to sequester the metallic ions of the nuclei.

The fact that unfixed resting hepatic nuclei are relatively homogeneous, despite a high content of polyvalent cations (7), has suggested the presence in these nuclei of a proportionally high content of negatively-charged metal-sequestering agents (22). These have been suspected to exist, not only in the form of soluble salts of electrolytes but also in the form of insoluble organic substances, such as desoxyribonucleic acid (2 a). The presence of acid proteins in nuclei (12, 20) suggests the possible involvement of proteins (21), as well, in the binding of nuclear cations.

SUMMARY

A simple, rapid method is presented for the isolation of nuclei from rat liver, with the use of glycerol and cation-binding reagents.

Nuclei showing three different types of optical appearance were isolated, depending upon the concentrations of the reagents incorporated in the glycerol solution. These nuclear textures have been designated as granular, homogeneous, and hyaline. This progressive increase in optical clarity has been interpreted in terms of the influence of metal-binding reagents upon the hydrated state of nuclear structures.

The present work emphasizes the role of negatively-charged ions in maintaining the homogeneous appearance of unfixed interphase nuclei.

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ELECTRON MICROSCOPE STUDIES OF ELASTIC TISSUE

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A KNOWLEDGE of the fine structure of elastic tissue is necessary for the understanding of many important pathological processes. In these laboratories, the characterisation of elastic tissue has been investigated by various methods and a general account of the findings has already been published (8). In addition, initial results of a study of the elastin/elastase system have been reported (7). The present paper deals with the problem from the structural aspect and concerns some electron microscopical studies of the tissue, particularly with regard to the action of trypsin and elastase.

In 1944, Wolpers (15), using material from mouse aorta and ox ligamentum nuchae, first established that unlike collagenous structures, elastic tissue showed no specific structural features, under the electron microscope. In order to obtain fibres of a suitable size Wolpers found it necessary to treat the tissue with pepsin. The mouse aorta showed thin, fenestrated sheets, with numerous short fibres protruding from their surface. These fibres were dense in appearance and gave no evidence of cross-striations or of fine fibrillar structure. The partially digested material from ox ligamentum nuchae revealed dense, broad fibres, bearing no cross-striations; no sheet-like structures were observed. Wolpers also reported that treatment of such dense, broad fibres with acid orcein, a stain considered specific for elastic tissue, revealed a finer fibrillar structure. Gross (5) confirmed the original findings of Wolpers and extended them by studying not only aorta and ligamentum nuchae, but also the elastin of carp swim bladder. Gross considers that all elastic tissue has essentially a similar structure, being composed of uniformly dense and ill-defined fibres, intermingled with those of collagen. Such elastic fibres show a tendency to split into fibrils lying parallel to one another, although in many cases they cross, forming a network of open

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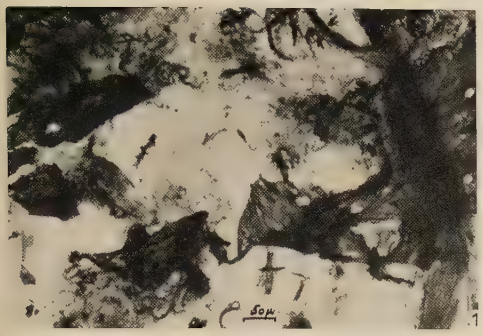
weave, the spaces of which are filled with an apparently amorphous matrix material. The latter, while resistant to heat, was easily digested by trypsin. His further contention that the ultimate structure of elastin was helical in nature has been disproved by Franchi and De Robertis (3 and 4) and Schmitt (12), who have shown this structural entity to be a contaminant derived from the trypsin itself. This has cast doubt on the views advanced by Gross as to the general architecture of the elastic tissue fibre. The elastic tissue of ox ligamentum nuchae has also been studied by Nutting and Borasky (11). After fragmentation of the tissue in a blender, the collagen was gelatinised by boiling for ten minutes in 0.1 *N* NaOH. The gelatin was removed from the preparation by solution in hot water and e.m. examination of the remaining material revealed no definite fibres; only loose reticular structures were observed.

It is clear therefore that much remains to be done before a general model of the architecture of elastic tissue fibres can be put forward. Wolpers's (15) study of aortic material for instance makes no mention of the two components observed by Benninghoff (2) in his histological studies (Fig. 1). This worker established that two distinct components, namely fibres and lamellae are present in human aortic media. The fibres lying between the lamellae are intimately associated with those of collagen and in morphology closely resemble the similarly staining regions of normal skin tissue. The lamellae are in the form of 'fenestrated sheets', that is, they contain holes; moreover, these sheets are of considerable extent and lie in roughly concentric layers around the lumen of the vessel. Benninghoff (2), however, found little evidence that they formed complete cylindrical sheaths. Gross (5) makes no mention of fenestrated sheets as a general component of aortic elastic tissue. The observation made by Nutting and Borasky (11) on ox ligamentum nuchae, that only reticular structures remain after the removal of the collagen component has not been confirmed; moreover, it conflicts with Wolpers's (15) findings and those of ordinary histology.

Fig. 1. Powder from human aortic tissue stained with resorinol-fuchsin, showing the fibrous and lamellar components.
Fig. 2. A typical dense fibre of elastic tissue from human aortic media, together with amorphous debris and cross-striated collagen fibrils.
Fig. 3. Elastic tissue from human aortic media after removal of collagen fibrils by treatment with acetic acid, showing dense branching fibres.
Fig. 4. Elastic tissue as in Fig. 3 showing dense fenestrated sheet-like structures.
Fig. 5. Portion of sheet as in Fig. 4, showing the presence of dense fibres protruding from the torn edge.
Fig. 6. Debris present in elastic tissue from human aorta.



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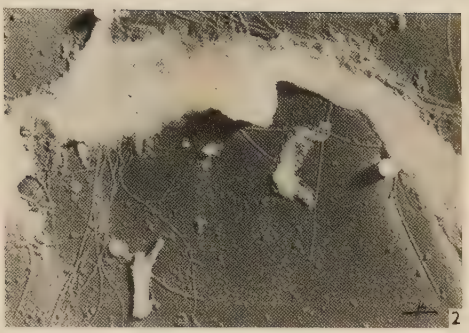
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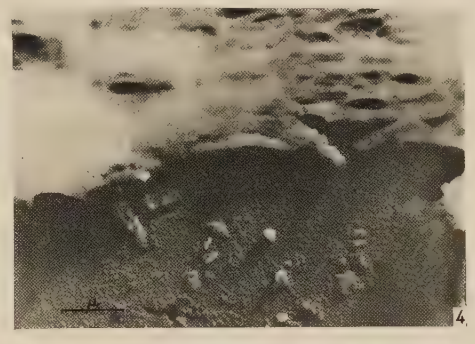
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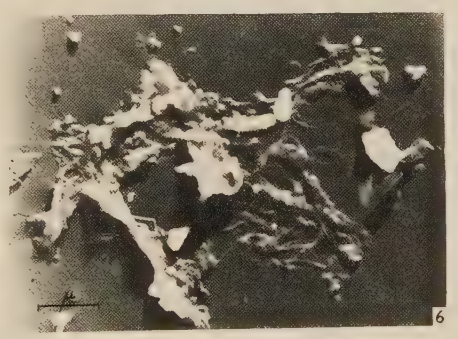
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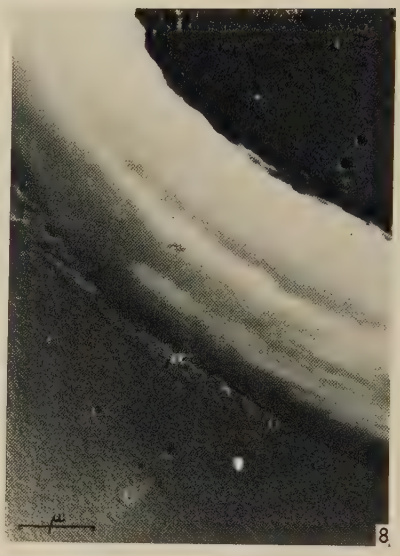

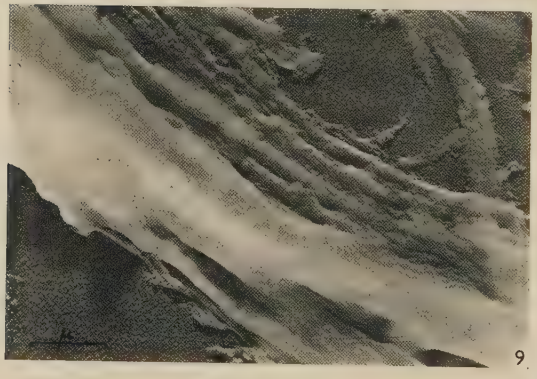

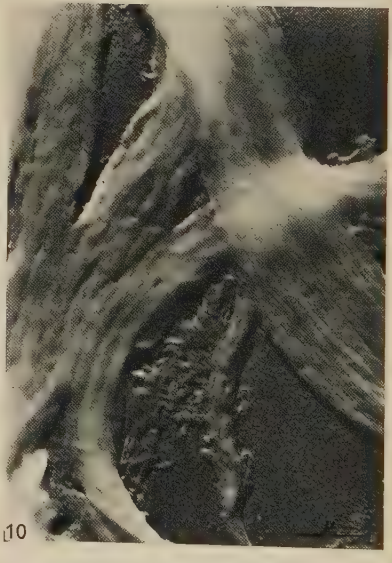

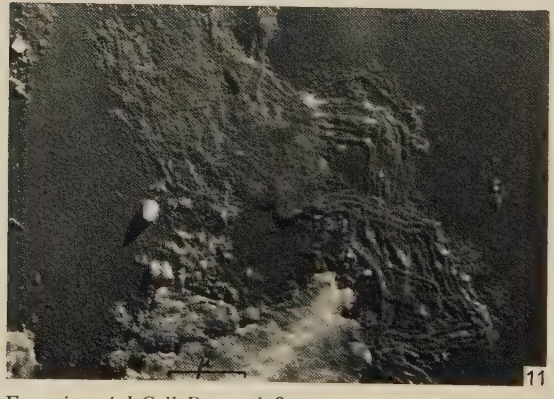

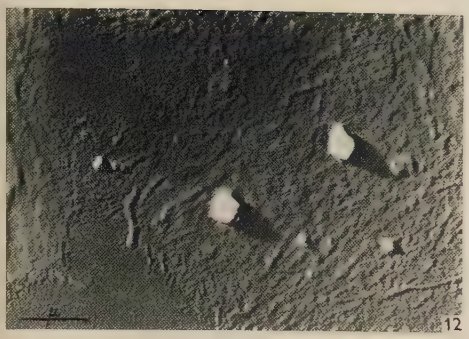

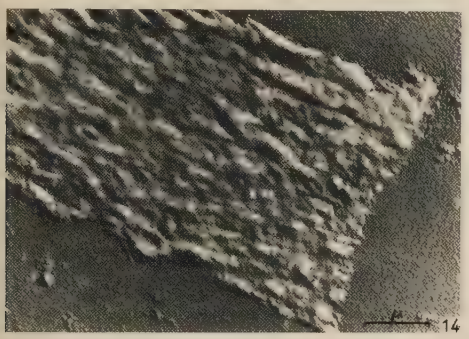

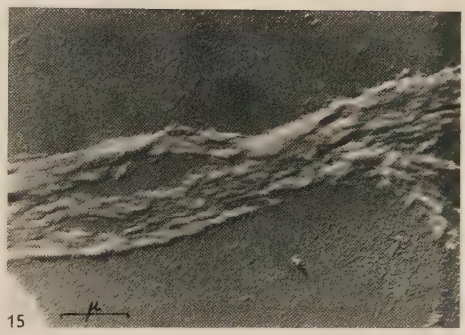

Fig. 12. As Fig. 10 and 11 elastase treated 90 minutes.
Fig. 13. As Fig. 10, 11 and 12 elastase treated 20 hours, showing the few corpuscles remaining.
Figs. 14 and 15. Fibres of ox ligamentum nuchae freed of collagen and treated with trypsin on elastase for the following periods.
Fig. 14. Trypsin 2 hours. Elastase 1 hour.
Fig. 15. Trypsin 2 hours. Elastase 4 hours.

MATERIALS AND METHODS

Human aortae, ox aortae and ox ligamentum nuchae were used as the sources of elastic tissue. A major difficulty in the study of elastic tissue is that of freeing the material from the collagen with which it is invariably associated. Another difficulty is the fact that the various forms of elastic tissue may have different chemical compositions. In previous papers (6, 8) we have discussed some of the factors involved in the isolation of a single chemical entity, elastin, from elastic tissue, and since this

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Fig. 7. Small sheet-like structure observed in aorta media.
Fig. 8. A fibre of ox ligamentum nuchae after treatment with acetic acid to remove the collagen.
Fig. 9. Fibres of collagen free elastic tissue from human aortic media after 2 hours treatment with a solution of trypsin at 37° C., showing little affect other than sharpening of the outlines.
Fig. 10. Collagen free elastic tissue after successive treatment with 1) trypsin for 2 hours and 2) elastase for 15 minutes.
Fig. 11. As Fig. 10 but after elastase reaction had proceeded for 30 minutes.

question is still unsolved, it is proposed to give full details of the treatment given to the samples examined.

Human aortae were collected at autopsy, at the latest within 24 hours of death. The body had been maintained at 0° C, and the samples after removal were stored in normal saline at −15° C. Since there was no evidence of degradation on storage, this method was employed as a routine procedure. The adventitial and the intimal layers were removed by dissection from those aortae showing no naked eye evidence of atheromatous degeneration. Material was obtained from subjects aged 5 months (foetal) to 55 years. The selected aortae were cut into small pieces and reduced to powder by mincing while frozen; finer powders were obtained by repeated maceration in a tissue blender in the presence of two volumes of ice cold 0.9 per cent saline. The powder thus obtained was filtered, washed repeatedly with ice cold water and extracted with acetone to remove lipoid material. Final drying with ether yielded a powder subsequently referred to as 'normal aorta powder'. When collagen-free material was needed, the powder was autoclaved for two hours at 10 lb./sq.in. pressure in three successive changes of 2 per cent acetic acid. Any collagen remaining after this treatment was removed by exhaustive extraction with boiling water until the effluent no longer gave a biuret reaction. The material thus obtained appeared completely free of the characteristically cross-striated fibrils of collagen when examined in the electron microscope (vide Fig. 3) and hence it was employed as starting material for the enzyme studies.

Ox aortae were obtained from healthy beef cattle at the local slaughter house, within half an hour of killing, and were treated in an identical fashion. Ox ligamentum nuchae similarly obtained was brought to a powder by macerating in the cold and freed from collagen in a manner similar to that used with aortic material.

The trypsin which has been employed was Armour's crystalline trypsin and was allowed to react at a concentration of 1 per cent on the tissue weight (the weight of enzyme includes 54 per cent magnesium sulphate) in 0.2*M* or 0.1*M* carbonate or phosphate buffer at pH 8.5.

The elastase was prepared by the method of Balo and Banga (1) and was allowed to react at 1 per cent concentration either at an initial pH of 10.3 in 0.1*M* glycine buffer or at pH 8.7 in 0.4*M* glycine buffer. The former pH was employed for the initial experiments with human aorta, but subsequent experiments made use of the observation (7) that the optimum pH for elastase action is in the region of 8.7 but that adequate buffering is necessary to prevent pH changes due to release of sulphuric acid—especially in the case of aorta elastin.

Where necessary the enzymes were destroyed after incubation at 37° by immersion of the reaction vessels in boiling water for 3 minutes. Two techniques were employed where treatment with trypsin was followed by that with elastase. In the case of reaction at pH 10.3, the solution was centrifuged after the inactivation of the trypsin, and the solid was resuspended in new buffer solution. In the case of the reaction carried out at pH 8.7, the same buffer solution was used throughout, and the trypsin after inactivation was allowed to remain in the system during the elastase reaction.

Electron microscope. For the e.m. examination of whole tissue, i.e. the tissue which had not been subjected to any chemical or enzymic treatment, small pieces were washed in distilled water and fragmented using fine sharp knives. Droplets of the resulting suspensions were placed on collodion-filmed specimen grids and after being

dried, were shadowed with chromium metal, following the vacuum technique of Williams and Wyckoff (14). In the case of tissue (powder) which had been chemically or enzymatically treated, the contents of the reaction flask were centrifuged and the sediment fixed either with 1 per cent formalin or with 1 per cent osmic acid solution (filtered). They were re-spun and washed with distilled water several times and after the final washing, the sediment was re-suspended in a small volume of distilled water to give a concentration suitable for e.m. examination. Droplets of the suspensions were mounted, dried and metal-shadowed as described above. During the electron microscope examination, many photographs were recorded from each sample, to obtain a representative view of the structures present. Following conventional e.m. practice, the prints shown are negative ones.

RESULTS

Human aortic media

The following components have been invariably observed in fragmented whole aortic media:—

1) the bulk of the tissue is made up of thick, dense, branching *fibres* (Figs. 2 and 3). Their widths vary, but most lie in the range of 1 to 4 μ; in length also they are not uniform, but are usually greater than 10 μ. Whilst some of these fibres have fairly straight edges, the majority are ragged in appearance, suggesting that they have been torn from their original position in the tissue. Generally, however, they appear to be roughly circular in cross-section as judged by length of shadow cast. Further, they appear to be coated with a dense material. They show no structural details such as cross-striation, but occasionally where the dense material is reduced in amount, smaller fibrils about $^1/_{10}$ μ across can be seen arranged in parallel fashion along the main axis of the fibre. The smaller fibrils, again show no cross-striations, and are best seen at the fraying ends of the larger fibres.

2) *large, sheet-like structures*, usually greater than 15 μ across (Figs. 4 and 5). These appeared to be composed of the thick, dense fibres described above. Some of these fibres are branched, others cross over in an irregular manner, and all are embedded in a dense interfibre material. Sometimes this material is absent and the sheets then manifest holes of irregular size and shape.

3) *debris material* of various sizes and shapes. From its ragged and indefinite appearance, this is probably interfibre material released from the sheet-like structures when the component fibres are torn out during the preparation of the sample for e.m. examination. Some of the more dense pieces, however, have angular shapes and are probably small sections of the thick fibres which form the major part of the tissue (Fig. 6). Occasionally small, thin masses of dense material are also observed; these are roughly

circular in shape, about 5 μ in diameter, and often have small holes about 1 μ in diameter (Fig. 7).

4) finally there are well defined *collagen fibrils* of uniform width, about 1100 Å and showing characteristic cross-striations (Fig. 2). These fibrils are present whatever the age of the tissue and they do not appear to vary greatly in amount in the age group studied. Although characteristically well-formed, the collagen fibrils were, however, significantly less numerous in the foetal samples.

The appearance of such a variety of structures is evidence for the complexity of the media of human aorta. All the components listed above, irrespective of the age of the sample, being invariably present.

Untreated tissue from ox aortic media

As seen in the electron microscope, this tissue is indistinguishable from that of the corresponding human sample. Thus large, dense fibres are observed, some embedded in sheets and held together by a dense interfibre material, together with much amorphous debris and numerous collagen fibrils.

Untreated tissue from ox ligamentum nuchae

Under the e.m. this tissue has a more definite structure than aortic material. Apart from a small amount of amorphous debris and numerous collagen fibrils, it appears to consist mainly of dense uniformly wide fibres (Fig. 8). Further, these are quite straight, lie parallel to each other and show no tendency to branch or anastamose. As distinct from the aortic tissue, there is no evidence that the fibres of ligamentum nuchae are associated with any sheet-like structures.

The action of acetic acid on elastic tissue

Human aortic media, ox aortic media and ox ligamentum nuchae were treated with acetic acid to remove the collagen fibrils and so assist in the e.m. characterisation of the remaining components. After this the remaining tissue structures were identical in appearance with those observed in the untreated tissues, only the cross-striated fibrils of collagen having been removed. This similarity in appearance after treatment with acetic acid does not necessarily imply that the remaining structures retain their original chemical identity, indeed there is evidence (8) that acetic acid does not remove all the chemically identifiable collagen from elastic tissue.

The action of trypsin on elastic tissue

Preliminary investigations of the action of trypsin on whole aortic media for periods up to 3 hours showed that enzymic action was mainly confined to removing the debris. The collagen fibrils appeared unaffected whilst the elastic tissue fibres were of cleaner outline owing to the removal of much of the loosely adhering debris (Figs. 3, 5 and 8). The action of the enzyme was studied more fully on tissue which had been freed from collagen by pre-treatment with acetic acid. Treatment for one hour with the enzyme proved sufficient to remove the bulk of the debris from the preparations and to produce elastic tissue of cleaner outline. The large sheet-like structures were also freed from adhering material and sharpened in outline (Fig. 9). Contrary to the findings of Gross (5) the prolonged action of trypsin (3 to 17 hours at 37° C) resulted in no pronounced attack on the elastic tissue components. The sheet-like structures were still observed even after 17 hours treatment, as were the dense, thick fibres and a very small amount of debris. Exactly similar results were found when collagen-free preparations of ox aortic media and ligamentum nuchae were examined.

Since trypsin appeared to have no profound effect on the elastic tissue preparations beyond cleaning them, treatment for one hour with trypsin was often used to obtain clearer electron micrographs in later studies on the action of elastase.

The action of elastase on elastic tissue

Human aortic media. The effect of elastase was observed on collagen-free tissue, after a preliminary treatment with acetic acid followed by trypsin. Samples taken fifteen minutes after the start of the reaction were similar in appearance to the starting material (Fig. 10). Thus the broad fibres, whether occurring separately or in the dense sheets were still present and apparently unchanged. The only sign of reaction was a significant decrease in the amount of loose debris, where this had not previously been removed by tryptic action. After thirty minutes, however, there was more evidence of attack; the amorphous debris had largely disappeared and a general loosening effect on the remaining tissue components was observed. The non-fibrous material appeared to be removed first by the enzyme; the large dense sheets were reduced to broad fibres and such of the latter as were originally present in the starting material, had flattened out to reveal a fine fibrillation stage (see Fig. 11). These fine fibrils were of uniform width approximately 200 Å across. The elastase was capable of further reaction, for the loose fibrillar structures derived from the originally dense, broad fibres, were reduced to

small corpuscles, approximately 200 Å in diameter (see Fig. 12). This effect of elastase was best seen in samples taken ninety minutes after the start of the reaction. The fine fibrillation stage of the collapsed fibres was no longer seen, and apart from a few partially attacked fibres small corpuscles alone remained (Fig. 13). Twenty hours after the start only a few minute corpuscles remained.

It should be stressed that the view outlined above for the course of the action of elastase is an overall one. Due to the size variation in the original tissue suspensions, not all the structures in the latter were attacked at the same rate. Thus fibres which were free and not contained in the sheet-like structures react more rapidly than those which have to be released from these structures by the action of the enzyme.

Ox aortic media. The course of reaction of elastase on elastic tissue from this source exactly paralleled that on the human material. Similar stages of enzyme attack were observed, and the whole sample was ultimately reduced to fine corpuscles.

Ox ligamentum nuchae. The action of elastase on ox ligamentum nuchae can be observed more easily than with aortic material. This is a consequence of the simpler structure of the ligament, the dense, broad fibres of which, unlike those from aorta, do not branch, nor are they embedded in sheet-like structures. Apart from this structural difference, however, the effect of elastase on the two forms of elastic tissue is very similar. The dense material adhering to the fibres was first removed (Fig. 14); the fibres collapse and parallel fine fibrils, lying parallel to each other, were revealed (Fig. 15). With prolonged action of the enzyme, the fine fibrils were ultimately reduced to small corpuscles, which mostly dissolved and brought about almost complete solution of the ligament.

It has been mentioned that the study of elastase action was carried out on material which had been pretreated with acetic acid and with trypsin. Preliminary investigations using elastase alone, on samples of whole tissue, showed that under the e.m. the collagen fibrils appeared to be quite unaffected by elastase, even after 8 hours contact with the enzyme at 37° C, during which period much of the elastin had dissolved.

DISCUSSION

As regards the structure of the untreated tissues, the results both confirm and extend the previous findings of Wolpers (15) and Gross (5). It is evident that aortic tissue has a more complex architecture than ligamentum nuchae; both human and ox aortic media contain large sheet-like structures, which

consist of thick, branching fibres, running in all directions and held together by a dense interfibre material. It would seem that not all the fibres present in aortic material are so contained in sheets, for in every sample examined many separate fibres were observed. Intimately associated with both sheet and fibre structures, however, are numerous collagen fibrils and probably much of the cohesion of the intact tissue is due to the underlying network of collagen fibrils. It has been shown that many of the sheet-like structures are partly devoid of interfibre material and display holes. Many of the fibres observed in normal elastic tissue appear to be roughly circular in cross-section, as judged by the length of the shadows cast in the metal-shadowed preparations. They show no characteristic or regular structure e.g. cross-striations, though many tend to split into thinner parallel fibres. None of the very fine fibrils observed in elastase treated material, were observed in normal (untreated) tissue. All the powdered forms of elastic tissue examined in the electron microscope contained large amounts of ill-defined debris, and it is impossible from the present work to decide whether such material was formed during the preparation of the samples for e.m. examination, or whether it was present as such in the intact tissue. The presence of so much debris material in the latter suggests that elastic tissue contains material with poor cohesional properties.

The tissue from ox ligamentum nuchae on the other hand has a much simpler architecture than the aortic media. No sheet-like structures are present; the only components observed in the electron microscope are dense broad fibres, which, showing no tendency to branch, are closely packed in a regular, parallel manner. Intimately associated with these fibre bundles are the smaller fibrils of collagen and as in the case of the aortic media, they may contribute to the stability and cohesion of the total structure. Much amorphous debris is also present, but again, it is impossible to decide its origin and whether or not it has any functional significance. Like the fibres of aortic tissue, ligament fibres occasionally show a tendency to split into smaller fibres, but only in the elastase treated material were very fine fibrils observed.

A comparison of elastic tissue from aorta and ligamentum nuchae shows that they have much in common. Both contain thick fibres, similar in appearance, associated with a dense amorphous material and with many collagen fibrils. Thus, if elastic tissue contain a common entity, elastin, then it would seem that such an entity must be dual in nature consisting of thin fibres, in close association with a dense, non-fibrous component. This hypothesis disregards the large amount of debris material invariably present in

powdered samples of elastic tissue; if such material is not a fragmented form of the two component structure already postulated, then possibly the complete elastin structure may be even more complex. None of the fibrous structures present in elastin appear to have any periodic structural feature such as cross-striation. This model for the elastin fibre implies that the cohesion of the fine fibrils in the thick fibres is determined by the non-fibrous component; in other words, the latter seems to function as a cement material. Even so, as far as can be judged from the electron micrographs, the fibres in both aortic and ligament tissue are not of themselves very rigid structures. The relative straightness of the ligament fibres compared with those from aortic tissue, suggests that the former are the more rigid. Thus while the cement material certainly aids in the cohesion of the fine fibrils to form thick, robust fibres, it seems inescapable that the cohesion of the tissue as a whole is largely determined by the underlying network of collagen fibrils.

Even if it is assumed that a common structural and chemical entity is present in both aorta and ligament, it is quite clear that the gross arrangement of the elastin fibres is quite different in the two tissues. In aortic media, the fibres show no regular, parallel arrangement, but run in all directions and with the aid of the cement material, elaborate large, sheet-like structures. In ligamentum nuchae, the fibres are arranged in a regular, parallel manner and no sheet-like structures are present. These facts provide a basis for explaining the differences in mechanical behaviour and function of the two tissues. Aortic tissue, as part of a vessel wall, would need to be elastic in more than one direction. On the other hand, the ligament needs to be elastic in one direction only, namely, along the axis of the ligament. The sheet-like structures in the aortic media, with their randomly arranged fibres, would show this type of elasticity, whereas the ligament, with fibres arranged in a parallel manner, would tend to exhibit elasticity in one direction only.

Regarding the action of trypsin on elastic tissue, Gross's 1944 observation that the dense coating material associated with the fibres is rapidly digested by this enzyme, is not confirmed. Further, his statement that prolonged tryptic action brings about the collapse of the fibres and releases pairs of spirally wound filaments is not borne out by the present work. It is significant that in Figs. 3 and 5 Gross's paper, showing the action of trypsin on elastic tissue, fibres are observed with the dense coating material still adhering to them. In our experience, treatment for as long as 17 hours with this enzyme produces no significant change in the fibres of elastic tissue, and this would indicate that if elastin, the common component of all elastic tissue, is indeed a pure protein entity, then it must in some way be well protected from the

action of proteolytic enzymes. In our view, trypsin digests only the amorphous debris invariably found in the powdered samples; the more intact fibres do not appear to be appreciably altered. Thus the action of trypsin on elastic fibres appears to depend on the physical state of the preparation. Since both elastase and trypsin digest the debris while elastase alone digests the intact fibres two possible explanations suggest themselves. Fragmentation of the amorphous portion of the fibres must render it more susceptible to attack either by the trypsin itself, or by a small amount of elastase present as a contaminant in the trypsin. Which alternative is correct is difficult to decide, although biochemical observations on the rates of reaction of both trypsin and elastase would tend to discount the contaminant theory. If this is so one must infer that fragmentation of the amorphous substance reveals protein structures which are labile to trypsin.

Elastase on the other hand has a considerable effect on elastic tissue. Its action is rapid and it brings about almost complete solution of the substrate. The non-fibrous component appears to be attacked first. The interfibre material of the large sheet-like structures is removed and the embedded fibres are rapidly released. At the same time, the separate fibres which may be present are stripped free from their dense coating of cement material. The step which determines the rate of solution of the tissue appears to be the removal of non-fibrous cementing material. Once this is removed, the fibres, which previously were robust, bulky structures, immediately collapse and form flattened systems of fine parallel fibrils. Apparently these fine fibrillar systems, of loose texture and free from the cement material, are very easily reduced to small corpuscular units. The fine fibrillation stage of breakdown is a transient condition for most fields examined in the e.m. showed signs of the further reduction to corpuscular units.

This breakdown of the fibre structure with elastase action has been observed with the three types of elastic tissue used in the present work. It therefore provides a basis for establishing a general model of the elastic tissue fibre. Fine fibrils, lying parallel side by side, are held together by means of a dense non-fibrous, cement material to form stouter fibres. Thus much of the cohesion of the elastic tissue fibre is due to the cement material. This model implies that the fibre is a two component system consisting of fine fibrils and a cement material. This view of the fine structure of the elastic tissue fibre, where the component fibrils lie parallel along the axis of the fibre follows naturally from the elastase studies, though it conflicts with Gross's suggestions that all forms of elastic tissue are based essentially on a small double spiral unit.

We have proposed on chemical grounds (8) that elastin is a complex structure containing two components, fine fibrils held together by a cement substance. Hall (7) in a study of the action of elastase has also found evidence of two components in elastic tissue, one of which is sensitive to the action of alkalis. It is possible that the fibrils and cement substances described in the present work may correspond with the chemical fractions referred to by Hall but further studies are necessary before the correlation can be assumed.

Since this work was completed reports have appeared from two schools of workers concerning the effect of elastase on elastin, as viewed under the electron microscope (10, 13). In the former report morphological findings are given which are essentially similar to those we have observed. Schwarz and Dettmer on the other hand report that after treatment with elastase, they observe striated fibrils in the intersheath areas of aorta. These they claim to be the inner fibrillary structure of elastin fibres. As has been suggested (9) we do not think that these two authors have taken adequate steps to ensure that all the collagen has been removed from these sites before the sections were examined under the electron microscope. Therefore although the periodicity of the fibres differs from that of collagen from other sources there is no definite evidence that the striated fibrils do in fact originate within the elastin fibres.

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CHANGES IN THE SUBMICROSCOPIC STRUCTURE OF THE CYTOPLASM ATTENDING MATURATION AND ACTIVATION OF THE SEA URCHIN EGG[1]

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This writer (21) made a number of observations indicating the presence of fibrous elements in the cytoplasm of the unfertilized mature egg of the sea urchin *Paracentrotus lividus*. For example after treatment of the eggs with a hypertonic solution rather long fibrous elements appeared in groups with a tendency to parallel orientation of the elements. Upon immersion of fertilized eggs in glycerin birefringent patches were observed (21).

Unfertilized eggs, on the other hand, may appear as positive spherites when examined between crossed Nichols upon immersion in glycerin, see Runnström and Monné (28).

These authors also continued the observations on eggs (of *Psammechinus miliaris*) subjected to a hypertonic medium. Fibrous structures appeared mainly on the border between a peripheral granular zone of the egg and a central zone relatively free of granular components. Monné (12, 14) subjected the eggs to centrifugation and showed that the fibrous elements appeared in the clear zone which is formed in the centrifugal part of the *Psammechinus* egg. Moreover he demonstrated that the fibrous elements are negatively birefringent with respect to their longitudinal axis. McCulloch (11) carried out observations on the fibrous structures in the egg of *Arbacia lixula*. Again he found that these structures belong to the ground-substance of the cytoplasm. In the *Arbacia* egg the clear zone appears in a more centripetal position than in the *Psammechinus* egg, see Harvey (5).

METHODS

The eggs of *Psammechinus miliaris* and of *Echinocardium cordatum* were used for the observations. The eggs were removed from the ovary and cleaned in the customary way (4). The birefringent fibrous structures were made to appear simply by putting the eggs in a drop of sea water between slide and cover slip and leaving them at

[1] Dedicated to Professor W. J. Schmidt, Giessen, Germany, on the occasion of his 70th birthday.

room temperature for 1–2 hours or more. In the eggs near to the border of the drop the birefringent fibres or rods appeared earlier than in eggs in the middle of the drop. Therefore the drop with eggs was often put into the concavity of a slide of the type used for tissue culture. The gradual increase of hypertonicity due to the evaporation seems to be favorable for the appearance of the birefringent fibrous structures. But these appeared also when the eggs were brought directly into a hypertonic solution. The sea water supplied to the aquarium of the Zoological Station of Kristineberg can serve as a hypertonic medium for the eggs of the littoral form of *Psammechinus miliaris*. The salinity of this water is about 32 per cent, whereas the water in the littoral zone has a varying salinity often around 26 per cent. In the course of 4–5 hours many birefringent fibrous structures appear in the egg. In another set of observations the eggs were subjected to a mixture of 9 ml sea water + 1 ml 2.5 N NaCl-solution. Here the fibrous structures were observed after 1–2 hours. However, the time necessary for making the fibrous structures visible varied considerably.

The observations were made with a Leitz polarization microscope. This was provided with an elliptical compensator with a retardation 0.033 λ.

RESULTS

The observations were mainly carried out on oocytes and mature non fertilized eggs.

Upon exposure to hypertonicity the birefringent fibrous structures appeared earlier in oocytes than in mature non fertilized eggs. In the oocytes the fibres appeared particularly in the neighborhood of the nucleus (germinal vesicle). Under the influence of the weak hypertonicity the nucleus changed its form. The vegetal surface became concave whereas the animal surface remained convex.

The birefringent fibrous material was often particularly conspicuous near to the border region between the convex and the concave part of the nucleus, see Fig. 1, or it was accumulated in the part of the cytoplasm protruding into the vegetal part of the nucleus, see Fig. 2. Fibres or rather thick rods were present also close to the convex (animal) border of the nucleus, see Figs. 1 and 2.

The birefringent material was often observed in the part adjacent to the vegetal border of the nucleus prior to its appearance in the animal region of the cytoplasm. The fibres or rods were negatively birefringent with respect to the longitudinal direction. In the region between the convex part of the nucleus and the cell surface sometimes a birefringence was observed which was not associated with visible fibres but with the ground substance. Such regions are indicated on Figs. 1 and 2 by finer parallel lines. The crosses outside these regions are meant to indicate that the birefringence in these regions is positive with respect to the radial direction of the oocyte. It

could not be settled whether these birefringent regions or patches have anything to do with the cytocentrum.

In oocytes suspended in normal sea water the cytocentrum can be distinguished as a hyaline spot. It has its position rather close to the animal surface of the nucleus. Parallel to the surface of the nucleus membrane certain strands are visible which present a radial arrangement with respect to the cyto-

Fig. 1. Oocyte of *Psammechinus miliaris* exposed to gradually increasing hypertonicity in a drop of sea water. Fibre- and rod-like birefringent structures appeared close to the nucleus. The cross with unequal axis indicates the sign of birefringence of a part of thecytoplasm in which no microscopic fibers were formed.

Fig. 2. Oocyte of *Psammechinus miliaris* exposed to gradually increasing hypertonicity in a drop of sea water. Similar to Fig. 1 but with many rod-like structures in the region of cytoplasm protruding into the nucleus.

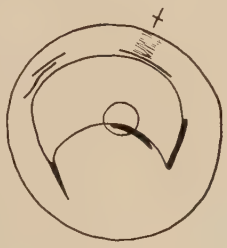


Fig. 1.

Fig. 2.

centrum. Submicroscopic fibrous or membranaceous structures are probably oriented parallel to the surface of the oocyte.

Only very rarely birefringent fibres were seen within the nucleus of the oocyte. Monné (13) has described the occurrence of such fibres from observations on fixed and stained material but their occurrence is certainly exceptional even in eggs subjected to weak hypertonicity.

The birefringent fibres which appeared in the mature egg were more spread over the whole cytoplasm, see Figs. 3–6. A certain tendency to parallel arrangement can be recognized. Different groups of fibres may be perpendicular or form other angles with each other.

Usually the fibrous elements appeared in a subcortical layer of the cytoplasm, as was earlier described (14, 28). The tendency to parallel orientation may possibly be due to slow streaming movements (14). Anyhow, it could be observed directly how the fibers or a certain part of them changed their orientation. They were first arranged tangentially but assumed gradually a more radial direction, see also (22, p. 282).

The formation of birefringent fibres in the mature egg is influenced by addition of sperm extract to the medium. Dense suspensions of sperm of *Brissopsis lyrifera* were centrifuged and the supernate removed and stored. The sedimented spermatozoa were resuspended in 32 per cent sea water and the same operation was repeated several times. In this way supernate 1, 2, 3 etc. were obtained, see (7).

Eggs of *Psammechinus* were suspended in *a*) sea water 32 per cent, *b*) in supernate 2, *c*) in supernate 3, *d*) in supernate 4. In the course of 3–4 hours a rich formation of birefringent fibres occurred in *a* whereas no such fibres were visible in *b*–*d*. On the other hand the presence of the sperm extract favored the activation of the eggs. This held particularly for *b* where all the eggs exhibited an activation membrane. The membrane was only moderately elevated from the egg surface. Moreover it was often granular. In *c*–*d* the number of activated eggs was lesser but no birefringent fibres were seen. In the activated eggs often a monaster appeared. This was later transformed into a "clear streak" exhibiting a negative birefringence with respect to the longitudinal direction, see Monné ((12), Fig. 16). In few cases a positively birefringent spindle or astrosphere was observed.

Albumin solution in sea water 0.1–0.2 per cent seemed to have a similar effect as the sperm extract in preventing the formation of the scattered birefringent fibers.

As found already by Monné (12, 14) the birefringent material accumulated upon centrifugation of the mature egg in the clear zone. This zone contains the basophilic small granules (12) and the ribonucleic acid (6). The clear zone may appear birefringent as a whole but in eggs subjected to hypertonicity rod-like birefringent structures were mostly recognized. The birefringence is negative with respect to a direction perpendicular to the axis of centrifugation.

The previously described rod shaped crystal within the nucleus of the mature egg was also found in the course of this investigation, see (13, 19, 20). The formation of this structure is also induced by weak hypertonicity. It is positively birefringent with respect to the longitudinal direction and confers an elongated form upon the nucleus (13). The nuclear birefringent rods only appeared in eggs in which the cytoplasmic structure remained comparatively homogenous. The cytoplasm did not exhibit the rod- or fibre-like structure. A certain birefringence could be observed in a subcortical region of the cytoplasm. This birefringence seemed to be bound to oriented submicroscopic fibres. The sign of the birefringence of the oriented streaks of the cytoplasm was negative. Eggs with nuclear birefringent rod were fertilized. The rod disappeared in connection with the dissolution of the nuclear membrane. Sometimes the rod was first broken up in a number of spheres. Each one of these presented a birefringence positive with respect to the radial direction.

It may be mentioned that in the non fertilized mature eggs subjected to hypertonicity positively birefringent spheres sometimes appeared besides the

birefringent rod- or fibre-like structures. These spheres are probably rich in lipids and may correspond to the myelin figures which arise in the egg under different circumstances, see (20). They were never observed in the cytoplasm of the oocytes.

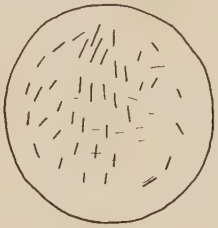
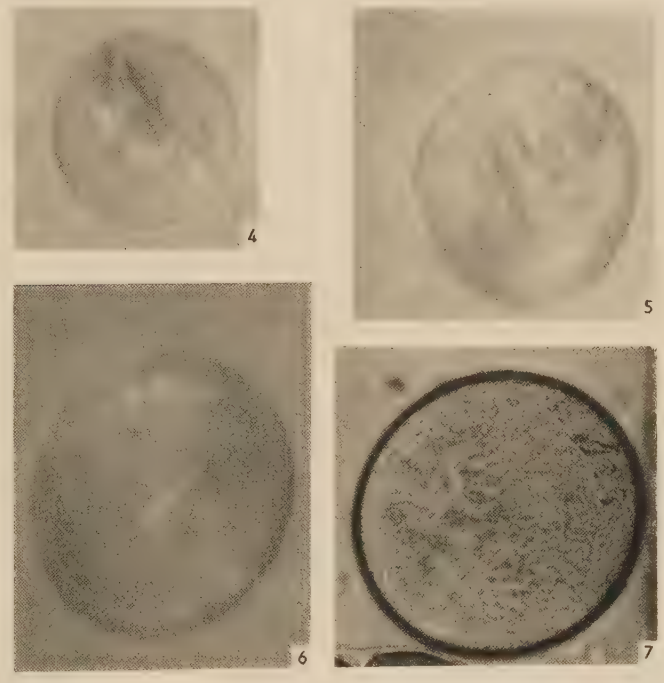


Fig. 3. Mature non fertilized egg of *Psammechinus miliaris* exposed to gradually increasing hypertonicity in a drop of sea water. Numerous rod-like birefringent structures with a tendency to parallel orientation



Figs. 4–6. Photos of non fertilized mature eggs of *Psammechinus miliaris* exposed to gradually increasing hypertonicity in a drop of sea water. The eggs were observed between crossed nichols with the elliptical compensator inserted. Numerous birefringent fibre- or rod-like structures in the cytoplasm. Fig. 7. Centrifuged non fertilized egg of *Psammechinus miliaris*, about two hours after centrifugation. Irregular bodies still accumulated at the centripetal pole; stratification otherwise almost completely reversed.

Even in normal medium the mature eggs sometimes contained rather numerous scattered bodies of varying configuration, see (21, Fig. 14). According to previous experience they are rich in lipids. Upon centrifugation these about 2 μ large bodies (Golgi apparatus?) moved in a centripetal direction, i.e. in a direction opposite to that taken by the mitochondria. Fig. 7

shows a number of the bodies mentioned accumulated in the centripetal part of a centrifuged egg.

It is surprising to see how certain batches of eggs are rich in the bodies whereas others are devoid of them. In no case, however, they were found to be spread in the cytoplasm of the oocytes. This again illustrates the striking differences between these and the mature eggs.

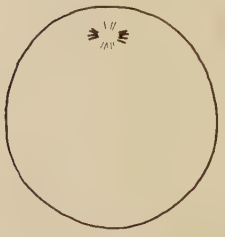


Fig. 8. Egg of *Psammechinus miliaris* undergoing the first meiotic division Positively birefringent astrosphere at one pole of the egg. The egg had been exposed to gradually increasing hypertonicity in a drop of sea water. In oocytes and mature eggs present in the same drop numerous birefringent rods or fibers were visible.

During the course of the observation on the effect of hypertonicity many single eggs were found to be in the stage of meiosis. In these a positively birefringent astrosphere was often visible, see Fig. 8. These eggs contrasted both with the oocytes and the mature eggs by the fact that no negatively birefringent fibres were present in the cytoplasm even if the eggs were exposed to hypertonicity. This was shown to hold irrespective of the way of bringing about hypertonicity.

The following observation was also made repeatedy both on eggs of *Echinocardium cordatum* and of *Psammechinus miliaris*.

Under the influence of evaporation the birefringent fibres appeared in the cytoplasm of mature non fertilized egg in the course of 60 90 minutes. Suddenly the elevation of an activation membrane occurred in single eggs. Sometimes this membrane was less refractile than a normal fertilization membrane; in other cases its properties were equal to those found in a normal membrane. The most striking feature of these observations was, however, that the conspicuous birefringent fibers completely disappeared in the eggs in which a membrane elevation occurred, see also (21, p. 211).

According to Kriszat and Runnström (8, 25) eggs pretreated with sodium periodate may undergo activation. Eggs of *Psammechinus miliaris* were subjected for 10 minutes to sea water with $5 \cdot 10^{-5}$ M periodate. The eggs were then transferred to normal sea water. Drops of the egg suspension in sea water was brought between slide and cover slip for slow evaporation. The pretreatment of the eggs with periodate sensitizes the egg to the activating

effect of hypertonicity. A varying number of eggs exhibited membrane elevation. In these eggs no birefringent rod- or fiber-like structures appeared. In the non activated periodate-pretreated eggs, on the other hand, a rich formation of birefringent rods or fibres occurred.

DISCUSSION

According to the data submitted above a prolonged exposure of oocytes or mature eggs to a hypertonic medium favors the appearance of birefringent fibre- or rod-like structures in the cytoplasm. It seems probable that these structures are preformed. The effect of the hypertonicity probably brings about an aggregation of submicroscopic fibrous elements. Such elements have in fact been demonstrated by the electron microscopic studies of McCulloch (9) on *Arbacia punctulata*. Also Afzelius (1) found fibrous or membranaceous submicroscopic structures in the egg of *Psammechinus miliaris*.

In the oocytes the birefringent fibrous structures appear preferently close to the nucleus. In the mature egg they have no relation to the nucleus. They are here accumulated primarily in a subcortical layer but may be present in the whole endoplasm. More rarely they are found in the cortex. The different localization of the birefringent structures may be due to the considerable rearrangements which follow upon the maturation of the egg, see (10, 16, 29).

It is probable that the localization may be determined by the fabric of the cytoplasm. Where the cytoplasm is rigid due to a continuous framelike gel-structure the possibility for a crystallization is lesser. This may rather occur in regions of lower rigidity. This view seems to apply rather well to the oocyte. An examination of various phenomena lead to the conclusion that the outer cytoplasm of the oocyte is constituted of a framework in which the elements are rather firmly linked together. The cytoplasm immediately surrounding the nucleus may have a lower rigidity. This assumption is in keeping with the separation of the oocyte into two regions that has been observed under different conditions (25), see also (27, p. 182). The space adjacent to the nucleus would thus offer lesser resistance to the crystallization process than the more peripheral part of the cytoplasm of the oocyte. In this at most an orientation on the submicroscopic level may occur. It is well known that after the maturation the rigidity of the egg cytoplasm decreases. The cytoplasm acquires a more clusterlike structure as contrasted with the continuous framelike structure of the oocyte cytoplasm (3). Accordingly the

capacity of formation of fibre- or rod-like structures increases. The cortex is the part of the non fertilized mature egg in which the rigidity of the cytoplasm is highest. Here the formation of microscopic fibre- or rod-like structures is rare. A certain orderliness of its submicroscopic structure is indicated, however, by the birefringence of the egg surface, for references see (22, p. 280).

In an attempt to reveal the nature of the birefringent fibrous- or rod-like structures the eggs were homogenized and subjected to centrifugal fractionation, see (7). After centrifugation 105,000 × g for 5 hours of the second supernate a sediment was obtained which consisted of a birefringent gel. Upon swelling it exhibited a fibrous structure. The fibers were positively birefringent with respect to the longitudinal direction. They were broken down upon addition of cryst. trypsin and may therefore mainly consist of proteins. Among the fibres granules were present that appeared very dark in phase contrast. They faded after treatment with ribonuclease whereas the fibres were resistant to this enzyme. The granules were probably constituted by groups of submicroscopic granules (microsomes) rich in ribonucleic acid. Similar granules can be recognized more vaguely also in the living cells e.g. within the astrospheres of cleaving fertilized eggs.

The identity of the fibres of the homogenate fraction with the intravital fibres seems to be obviated by the different sign of birefringence. On the other hand the fibres of the homogenate agree with respect to the sign of birefringence with the spindle and the astrospheres.

The strong centrifugation may have been able to separate a cover of oriented lipid molecules from a core of protein fibers. In this way a change of the sign of birefringence might have been brought about.

It seems probable that the microscopic fibres under discussion are formed secondarily by an aggregation of smaller components.

The relatively rapid formation of the clear zone upon centrifugation of the intact egg indicates that it consists of continuous structures of microscopic dimensions rather than of free submicroscopic particles. Otherwise it would hardly be possible to separate out the clear zone by the relatively slight accelerations adopted (e.g. 5,000 × g for 30 minutes). When a stratified egg becomes fertilized and enters into a diaster stage the axis of the spindle often coincides with the former axis of centrifugation. The "centrifugal" astrosphere is more strongly developed than the "centripetal". The former grows stronger by incorporation of the material constituting the clear zone. In phase contrast one can see how the "centrifugal" astrosphere is considerably richer in dark granules than is the "centripetal". One cannot avoid the

impression that there is a relation between the "dark" ribonucleic acid containing granules and the extension of the astrosphere. They are both derived from material which upon centrifugation accumulates in the clear zone.

The main new result of this report was the statement that negatively birefringent fibre- or rod-like structures never appeared during the meiotic divisions. The small positively birefringent astrosphere observed in connection with the meiotic division in no way corresponded to the rich development of birefringent structures in primary oocytes or mature eggs exposed to hypertonic medium. The properties of the cytoplasm seem to change during the meiotic division in such a way that no precipitation of fibres occurs upon exposure to a hypertonic medium. This is to compare with the disappearance of the birefringent structures in connection with the membrane elevation which sometimes occurs in the hypertonic medium, particularly subsequent to pretreatment with periodate. The membrane elevation indicates an activation of the egg. The activated cytoplasm reacts to the hypertonic medium in a way similar to that found during the meiotic divisions. Kriszat and Runnström (9) already pointed out that fertilized eggs subjected to hypertonicity showed no formation of scattered birefringent rods or fibres.

On the other hand, the whole cytoplasm has the potentiality of exhibiting double refraction as follows from observations referred to in the introduction to this paper, see (21, 28). In the non fertilized eggs immersed in glycerin the spherite structure was probably due to tangentially arranged submicroscopic fibres or membranes which form a part of the ground cytoplasm. Microscopic rods and fibres only appear when the"crystallization process" is slow as for example when occurring as a consequence of a gradually increasing hypertonicity. Kriszat and Runnström (9) found that fertilized eggs pretreated for 30 minutes with colchicine in the unfertilized stage are also much more resistant against the dehydrating action than are non fertilized eggs. After a somewhat prolonged treatment, however, negatively birefringent rods appeared in many eggs. A closer examination revealed that the rods were present in eggs in which the fertilization membrane was absent or incomplete. In colchicine pretreated eggs with normally elevated membrane the egg structure remained homogenous.

Previous to these observations experiments were carried out that seemed to demonstrate a greater "stability" of the cytoplasm of fertilized egg as compared with that of the non fertilized egg (21). The greater stability was assumed to depend on the transition from a clustered structure to a more dispersed three-dimensional framework having a higher degree of hydration. This conception is in keeping with the dissolution of the condensed

birefringent structures upon activation of eggs exposed to the hypertonic medium.

Recently emphasis has been laid on the role of particulate components in different steps of the activation process (7, 17, 23, 30). The sudden disappearance of the birefringent structures in unfertilized eggs subjected to hypertonicity might be due to the release of a substance having a dispersing action on these structures. An extract from the fraction of microscopic granules of the egg sensitizes the egg to the action of a hypertonic solution (30). A membrane elevation occurs more frequently than in absence of the extract. Certain fractions of sperm extract act in the same way as the extract of the granular component of the egg. As shown above such sperm extracts counteract the formation of the rod- and fibre-like structures. Albumin had a similar action. It is known that calcium breaks up the granular components of the egg, see for references (23). Possibly a prolonged action of the hypertonic medium brings about an increase of the calcium concentration. When this reaches a certain threshold value the break up of certain particulate components occurs and the "dispersing" substance escapes into the surrounding ground cytoplasm. Periodate sensitizes the egg to the action of the hypertonic medium (8, 25). This was confirmed above. It seems probable that the periodate treatment lowers the threshold for the break up of the particulate components by calcium or rather replaces calcium in this respect (8).

The meiotic divisions also create a "dispersed" state in the cytoplasm which is refractory to the condensing action of a hypertonic medium. In view of the previous consideration this would probably mean that a break up of particular components has occurred with the subsequent release of substance. This interaction between particulate components and the ground cytoplasm may represent a general occurrence in connection with cell divisions. One may remember the results reported by Chèvremont and Fréderic (2). With the help of phase contrast these authors studied mitoses in muscle cells or fibroblasts from chicken embryos. They were able to state that a reduction in size of the mitochondria occurs in the course of the pro- and metaphase. Certain mitochondria may even partly dissolve. After completion of the mitosis the mitochondria increase both with respect to size and number.

Porter (18) made a combined light and electron microscopic study of cultured chicken or mammalian cells. He has paid particular attention to a complex reticulum of strands which he refers to as "the endoplasmic reticulum". This is to be identified with the basophilic component of the cytoplasm. Thus it has probably a high content of ribonucleic acid. The endo-

plasmic reticulum is supposed to be embedded in a more homogenous matrix. Obviously the endoplasmic reticulum corresponds to the material in the sea urchin egg which upon centrifugation accumulates in the "clear zone".

Of greatest interest is the statement that the reticulum can be coarser or more finely subdivided according to the physiological conditions. The system is finely reticulated in cells from young cultures. The fine reticulation is correlated with a minimum of Brownian movement and a greater degree of gelation. This corresponds also to the state prevailing in the sea urchin egg in connection with the meiotic divisions or after activation. Porter points out that there is a correlation between the arrangement of the strands of the reticulum and of the mitochondria. The same applies to the sea urchin egg. In the mature sea urchin egg the mitochondria are aggregated what reflects an aggregated state of the underlying "endoplasmic reticulum" (see (21), Fig. 2a, b and Fig. 11). Vacuoles may sometimes be distinguished in the aggregates or "clusters". The aggregated state of mitochondria has since been demonstrated more clearly by an electron microscopic study (1). The uniform distribution of the particulate components in the activated egg, on the other hand, is correlated with a more dispersed state of the ground substance, particularly the "endoplasmic reticulum". In a great part of the cytoplasm of the oocyte a similar state prevails.

Monné (15) has given a number of pictures which demonstrate very well the variations of the texture of the basophilic reticulum. Of particular interest are the results he obtained in eggs subjected to 0.2 N azide solution in sea water. The whole reticular material may here be converted into strand or rods with a periodic arrangement of the basophilic material. The respiration was not measured but it is not probable that the azide suppresses the oxygen uptake in non fertilized eggs. On the other hand, it may suppress the formation of energy rich phosphate bonds.

Porter (18) found in the epitheloid macrophages an orientation of the reticulum and of the mitochondria along radii from the cytocentrum. In a similar way the ground cytoplasm along with the mitochondria is radially arranged around the cytocentrum of the sea urchin oocytes.

Presumably the cytoplasmic reticular framework plays a great role for the cleavage pattern. The latent differentiation of the micromeres for example is certainly initiated by an accumulation of ground cytoplasm at the vegetal pole of the egg. According to Runnström and Kriszat (26) addition of ATP restores the cleavage pattern to the normal in an egg material in which an irregular and incomplete cleavage prevails.

It seems possible that ATP plays a certain role in the control of the state of the cytoplasmic framework, see also (24) and the above discussion of the effect of azide (15).

SUMMARY

The formation of fibre- or rod-like structures is described to occur in oocytes or mature non fertilized eggs of *Psammechinus miliaris* and *Echinocardium cordatum* when these cells are exposed to hypertonicity. The structures are negatively birefringent with respect to their longitudinal direction, see also (12, 13).

In the oocytes the birefringent structures appear preferentially close to the membrane of the nucleus. In the mature eggs the birefringent structures often have a subcortical position but they may be scattered over the whole cytoplasm. They are generally tangentially orientated. Streaming movements may gradually bring about a more radial arrangement.

In eggs undergoing meiotic divisions no formation of negatively birefringent structures occurs. In single mature eggs a membrane elevation occurred under the influence of the hypertonicity of the medium. In such eggs the birefringent structures suddenly disappeared. Pretreatment of the eggs with sodium periodate ($5 \cdot 10^{-5}$ M) in sea water increased the number of eggs exhibiting membrane elevation. In the eggs with no membrane the content of birefringent structures was high.

The phenomena occurring in connection with the membrane elevation are discussed in the light of newer results. The disappearance of the birefringent structures is assumed to be due to the release of a substance from particulate components. This substance has a dispersing effect on the ground cytoplasm. Similar interactions may occur also during the meiotic divisions. Comparisons are also made with results reported by Porter (18) for cultured chicken and mammalian cells. The changes in the "endoplasmic reticulum" according to the physiological state present striking analogies to cytoplasmic changes occurring in connection with maturation or activation of the egg.

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CYTOCHEMICAL REACTIONS OF NORMAL AND STARVING *AMOEBA PROTEUS*

I. LOCALIZATION OF THIOLS, DNA, RNA AND OTHER ORGANIC PHOSPHATES[1]

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Amoeba proteus has been extensively studied so that there is available considerable information about the morphology and the physiology of both normal and experimentally manipulated amoebas. There are, however, few cytochemical data concerning this organism. With this in mind, cytochemical localizations were carried out of the following organic entities: sulfhydryl groups, ribonucleic acid, desoxyribonucleic acids, and other organic phosphates. The alterations in the concentration and distribution of these constituents (with the exception of organic phosphates) during starvation were investigated.

MATERIALS AND METHODS

Handling of living amoebas. Amoebas were cultured using a modification of Brandwein's procedure (6), maintained at 18° to 20° C, and subcultured every four weeks. The agar base described by Brandwein was omitted from the culture bowls in order to avoid the possiblity of it appearing as a contaminant in the cytochemical studies. Healthy, well-populated cultures were produced in the absence of the agar base. Control material was taken from such cultures.

For the starvation series, amoebas were washed with fresh Brandwein solution until free of food organisms. Equal numbers were then pipetted into finger bowls (capacity, 350 ml) to which were added approximately 100 ml Brandwein's solution. These cultures were then stored in a refrigerated incubator. The cultures, maintained at 18° to 20° C, were examined daily and the debris, such as cytolyzed amoebas and encysted food organisms, was removed with a pipette. Periodically, amoebas were removed and prepared for cytochemical determinations.

[1] Taken from a thesis presented to the Faculty of the Graduate School of Arts and Science, New York University, in partial fulfillment for the requirement for the degree of Ph. D. in the Department of Biology.

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General Procedures

Fixation: The fixative used for all the cytochemical procedures unless otherwise indicated, consisted of 1 part neutral formalin and 9 parts of absolute ethanol. The choice of this fixative derived from several considerations: 1) its rapid penetration into the amoeba, resulting in instantaneous killing and fixation; 2) the demonstration of fine cytological detail in sections and whole mounts of amoebas fixed in this manner; 3) excellent preservation of the fixed material as indicated by observations for over a two year period. No significant differences were noted between amoebas kept in this fixative from a fraction of a minute to 24 hours. In the experiments described below, amoebas were fixed for 1–3 minutes.

Imbedding amoebas for sectioning: Specimens were fixed, dehydrated, cleared, and imbedded in a dish (glass balance rest) having a small conical depression, while observing them under the dissecting microscope. Fluids were added and removed by pipette (15).

Preparation and preservation of whole mounts: Amoebas were fixed, washed with absolute ethanol and hydrated to 70 per cent ethanol in a depression slide while being observed under a dissecting microscope. The amoebas in 70 per cent ethanol were then picked up with a pipette and dropped onto an albumin coated cover glass. At this point the material was either used directly for cytochemical testing or was stored until the termination of an experiment so that the testing of amoebas starved for varying lengths of time could be carried out simultaneously. For storing, the amoebas were dehydrated and imbedded in a thin film of paraffin while on the cover glass (15).

Preparation of internally stratified amoebas: Amoebas in Brandwein's solution were sedimented and their internal components stratified by one centrifugation for a total of 6 minutes, 1 minute at 300 × G followed by 5 minutes at 2000 × G. The supernatant culture medium was then quickly pipetted off and the fixative added. Using the same procedure of sedimenting by centrifugation and pipetting off the fluid, the amoebas were either hydrated as far as 70 per cent ethanol and then pipetted on albumin coated cover glasses and handled as described above, or completely hydrated and treated with reagents in the tube (*e.g.* normal amoebas tested for sulfhydryl or phosphate groups).

General Cytological Staining

Sections were stained according to the Heidenhain's iron hematoxylin procedure as modified by Hsu-Chaun Tuan (26).

Stratified whole amoebas were treated by a modification of the Regaud's method for the demonstration of mitochondria.

Cytochemical Procedures

Desoxyribonucleic acids. Whole mounts as well as sectioned amoebas were treated according to the Feulgen technique, in which the leucofuchsin (Schiff's reagent) was prepared by bubbling SO_2 through a solution of basic Fuchsin as described by Rafalko (24), with minor modifications suggested by Lessler (17). Sections were hydrolyzed for 10 minutes and whole mounts for 12 at 55° C in 1N HCl.

Ribonucleic acids. Amoebas (whole mounts and sections) were stained for ribonucleic acids as follows: 1) in 0.1 per cent Azure A according to the description of Flax and Pollister (11); 2) in pyronin B (0.25 gm in 100 ml McIlvaine's buffer, pH 4.4); and 3) in saturated aqueous toluidine blue for ten or twenty minutes, followed by washing and then differentiation for five minutes in 95 per cent ethanol. Description of the methods used in evaluating the specificities of these dyes has been deferred, with the purpose of greater clarity, to the section on results.

Organic phosphates. The procedure involved hydrolysis in a strongly acid ammonium molybdate solution as described by Angeli (3). Whole amoebas were incubated for 48–72 hours at 37° C in the hydrolysis mixture. The visualization of the precipitated ammonium phosphomolybdate was carried out essentially according to the description of Serra and Quieroz-Lopes (25).

Sulfhydryl groups. Chalkley's modification (7) of the Giroud and Bulliard's nitroprusside test for sulfhydryl groups was applied to living as well as whole (fixed) and sectioned amoebas. For total bound sulfhydryl groups, amoebas were treated with 10 per cent trichloroacetic acid prior to application of the nitroprusside test.

Special Procedures

Three starvation series, designated as A, B and C, were carried out. The first two of these series (A and B) terminated after thirteen to fourteen days of starvation, and the third (C) after twenty-two days. On alternate days, amoebas from the A and B series were examined under the compound microscope, and samples were removed at random and imbedded in paraffin for sectioning.

These sections (4 μ) were treated with: 1) the toluidine blue test for ribonucleic acids; 2) Heidenhain's hematoxylin; or 3) the Feulgen test for desoxyribonucleic acids. Sections of the A series were mounted consecutively on three cover slips in the following manner. The first section of a particular day of starvation was placed on cover glass number 1, the second section on cover glass number 2, and so on. The fourth section was then placed on cover glass number 1, and so on. The first cover glasses of each day of starvation (bearing sections 1, 4, 7, etc. were tested together with toluidine blue; the second cover glasses stained with Heidenhain's iron hematoxylin, and the third subjected to the Feulgen test. In series B, the sections of the amoebas which had been starved for different lengths of time were placed on single cover glasses. The sections of amoebas starved 2, 6, and 11 days were on one cover glass, those starved for 1, 8, and 14 days on another and so on.

In series C whole mounts were prepared on alternate days, preserved, and set aside until the series was completed. These whole mounts of amoebas starved for varying lengths of time were simultaneously treated with: 1) the Feulgen reagents; 2) toluidine blue; or 3) Chalkley's modification of the nitroprusside test for sulfhydryl groups (on amoebas pretreated with 10 per cent trichloracetic acid).

RESULTS

Fixation. The cytological picture resulting from rapid fixation with ethanol-formalin is excellent. The inclusions appear to be intact and undistorted

(with the exception of the mitochondria, which are probably distorted by the lipid-extracting action of the alcoholic fixative). The appearance is strikingly life-like (Figs. 1, 2, 7 and 8).

Other alcoholic fixatives including Carnoy's ethanol-acetic acid mixture or tertiary butanol-formalin were determined to be adequate fixatives. Poor results were obtained with 10 per cent aqueous formalin (buffered or unbuffered).

When observed under the dissecting microscope, the killing and fixing of amoebas by alcoholic fixatives was instantaneous. When aqueous formalin was applied, however, the amoebas were observed to stream and form abnormally large pseudopodia for several minutes, indicating that killing was not instantaneous. This throws doubt on the validity of cytochemical localization in amoebas treated with aqueous fixatives.

Observation of living starved amoebas. Observations of the alterations in living amoebas starved for 1 to 22 days are in agreement with those of Mast and Hahnert (22) and Andresen (1). Most noteworthy of these alterations are: 1) a progressive decrease in both nuclear and cytoplasmic volume, more pronounced in the latter; 2) a progressive decrease in mitochondria; 3) a progressive concentration of the refractive bodies and crystals; and, during the terminal stages, a cessation of movement followed by rounding and then cytolysis.

Alterations were noted in pseudopodial formation which were not described by these authors. An occasional abnormality in pseudopod formation was noted in amoebas starved for five days. With progressive starvation, more and more amoebas demonstrated atypical movement. One abnormality appeared to involve the breakdown of the gel at the base of the forming pseudopod which resulted in a spasmodic flow of cytoplasm. In other amoebas, there was noted a spatulation or widening of the advancing pseudopodia. These derangements in pseudopodial formation seem to indicate an incipient breakdown in the sol-gel processes underlying locomotion and are probably the early indications of the processes which result in cessation of movement, rounding and finally cytolysis.

General cytological stains. The nucleoli in sectioned nuclei appear as peripherally located, distinct black spheres stained with Heidenhain's iron hematoxylin, in contrast to the more lightly stained karyosome. The cytoplasm is pale grey, its vacuolar nature delineated and undistorted. Crystals, and on occasion, refractive bodies are seen but the alpha and beta granules are not apparent.

At 2 days of starvation, the nucleoli are like those of the controls. From

4 days on, several tendencies, not necessarily concurrent, were noted. These included; elongation of the nucleoli with a suggestion of end-to-end fusion, increase in volume, and decrease in the intensity of staining. The increase in volume need not be accompanied by a decrease in staining.

Cytochemical Localizations

Desoxyribonucleic acids. The application of the Feulgen technique to sections and whole mounts indicate that desoxyribonucleic acids are found exclusively in the karyosomal portion of the nucleus. The karyosome appears to be made up of a fine thread-like tangled network with granules along its length, both components being Feulgen positive. These observations are in agreement with those of Bogdanowicz (3 a), Chalkley (8), and others.

There is a progressive condensation of the overall karyosome as well as a concomitant increase in its overall staining intensity in starving amoebas. These observations are in agreement with those of Brachet (5). In whole mounts of amoebas starved for one to three days the karyosomal appearance was observed to be essentially similar to that of the controls. Elongation of the karyosomal granules was seen at five to seven days of starvation followed by an enlargement and rounding of these granules between nine and thirteen days. From the thirteenth day of starvation, this particular configuration, *i.e.*, the large round granules, apparently reduced in number, remained constant in shape and size. The intensity of the Feulgen reaction in these karyosomal granules was either greater than, less than, or equal to that observed in the karyosomal granules of amoebas during the first day of starvation.

In view of the apparent reduction in the number of karyosomal granules, it seems reasonable that the observed elongation was the result of a fusion of karyosomal granules, and that the enlargement and rounding involved the completion of this process. Since the granules are enlarged, it is possible that despite the reduction in number there is no actual change in the total volume of the karyosomal material. If this, and the absence of radical alterations in the staining of granules are considered, it seems likely that there may be no actual alteration in the amount of the karyosomal material during starvation.

Ribonucleic acids. The results from the application of toluidine blue, azure A, or pyronin to otherwise untreated amoebas (sections or whole mounts) were similar. Toluidine blue indicates ribonucleic acids to be present in: 1) the cytoplasm (pale blue), 2) the nucleoli (bright blue), as well as 3) the karyosome (pale blue, like the cytoplasmic color). The refractive bodies stain

metachromatically (red or purple). The color distribution seen in azure A-stained amoebas is the same except for a darker blue coloration throughout. The metachromatic refractive bodies stained by azure A are lavender. In both azure A and toluidine blue-stained amoebas the refractive bodies were frequently enlarged. Staining with azure A produced gross cytological artefacts in both sections and whole mounts. With pyronin, the cytoplasm is pink, the nucleoli red (not in as striking a contrast with the cytoplasm as in the previous tests), and the refractive bodies are an intense red (neither metachromatic nor enlarged).

The specificity of each of these dyes was tested in thin (4 μ) sections by using each of the following procedures prior to staining: 1) incubation at 60° C for varying lengths of time (1 minute to 24 hours) in a 100 mgm per cent solution of crystalline ribonuclease (Kunitz preparation) made up in McIlvaine's buffer pH 6.8; 2) treatment with 5 per cent and 10 per cent hot (90° C) trichloracetic acid for 15 minutes (a treatment which according to Pollister and Leuchtenberger (23), both bydrolyzes and extracts nucleic acids); and 3) incubation in 1 N HCl at 60° C for varying periods of time (9). In each instance, water or buffer controls were used at the indicated temperature.

Sections whose nucleic acid content had been completely extracted in the several ways indicated above, exhibited a pale cytoplasmic reaction in response to staining with pyronin and azure A, and no color reaction with toluidine blue. In each instance there was no nuclear coloration after the extraction. Thus, it would seem that in the amoeba, under the conditions used, toluidine blue is more specific for ribonucleic acids than either pyronin or azure A which appear to stain some other acidic compounds of the cytoplasm as well as the ribonucleic acids. An attempt was made to determine the nature of these cytoplasmic compounds.

The possibility of nucleotide staining was eliminated. Sections, incubated in cold 10 per cent trichloracetic acid (a treatment which according to Brachet (4), removes nucleotides from the sections) appeared only slightly, if at all, less basophilic than the controls. The possibility that an unextracted lipid might contribute to the basophilia (observed in pyronin and azure A staining) was tested by treating sections in ether or ether-alcohol for 24 to 48 hours prior to staining. In general, these sections appeared to be more intensely stained than sections not so extracted. This suggests that there is some masking (probably physical) of nucleic acids by lipids.

In view of the proved specificity of toluidine blue and lack of specificity of azure A and pyronin under the conditions used, only toluidine blue was

used to demonstrate ribonucleic acids in starving amoebas. The cytoplasmic reaction for ribonucleic acids on whole mounts of amoebas starved for one day is the same as that of the controls. A slight increase in intensity of staining was noted on the third day. From the fifth through the thirteenth day there is a progressive decrease in cytoplasmic staining, reaching a minimum at 13 days. This minimal level remains constant through the twentieth day. From the thirteenth day on, the cytoplasm is devoid of color (*cf.* Figs. 1 and 2). The observations made on sections are in agreement with the above.

The following changes were observed during the starvation period in the refractive bodies stained with toluidine blue: 1) a tendency for the refractive bodies to become swollen in the toluidine blue; 2) a progressive increase in the staining intensity; and 3) a progressive decrease in the water solubility of the refractive bodies.

In the first four days of starvation the refractive bodies stained with toluidine blue appear normal in size or slightly enlarged. Around the sixth day, more and more of the refractive bodies are swollen. From this day on, the trend for the refractive bodies is to become smaller and more intensely stained. There is considerable variation in this trend, however, since some amoebas at thirteen days of starvation have only "pycnotic" refractive bodies, while others at nineteen days have a considerable portion of their refractive bodies swollen (*cf.* Figs. 3 and 4).

In whole mounts, extracted for 24 hours in water at room temperature prior to treatment with toluidine blue, a differential solubility of the refractive bodies was noted in amoebas starved for different periods of time. In amoebas starved for one day, this extraction resulted in the virtual disappearance of the refractive bodies; in amoebas starved for three to seven days, the refractive bodies were either small, reduced in number, or entirely absent; and in those starved for nine to twenty days, the refractive bodies were not only dark and distinct, but generally larger and more numerous than in the earlier part of the series (*cf.* Figs. 5 and 6).

Mast and Doyle (21) describe the refractive body as having an outer layer which is a protein stroma impregnated with lipid. It is this portion which stains metachromatically. Internal to this, they describe a carbohydrate shell and innermost, an aqueous "core" of unknown composition. This inner aqueous portion contains a high concentration of salts which appear to remain unaltered during starvation (unpublished data). In view of this, the observed changes in volume of the refractive bodies stained with toluidine blue are probably not related to this internal salt solution. When one considers the other alterations, *i.e.*, the increasing intensity of the meta-

chromatic staining of the refracive bodies as well as their decreasing dispersibility in water with starvation, it would seem that the underlying cause is related to alterations in the outer portion of the refractive bodies.

In view of the lack of information concerning: 1) the action of the fixative in extracting lipids from the refractive bodies; 2) the variations or lack of variations in the lipid or non-lipid of the outer portion of the refractive bodies, as well as 3) the possible involvement of the inner carbohydrate shell in these phenomena, it is not possible to make a satisfactory hypothesis concerning the mechanism underlying the observed alterations. It should, however, be emphasized that these observed phenomena do suggest a participation of the refractive bodies during starvation, which has not been indicated in other studies.

In the nuclei of amoebas starved for one day, and stained with toluidine blue, the nucleoli appear as they do in controls. The nucleus of the amoeba starved for three days is essentially the same except that the nucleoli are slightly larger. From the fifth day on, the nucleoli are noticeably larger and slightly metachromatic, reaching a maximum between nine and thirteen days (*cf.* Figs. 3 and 4).

In amoebas extracted for 24 hours in water at room temperature prior to staining with toluidine blue, the nuclear picture was similar except that the metachromatic response appeared earlier, on the third day, and was more intense from then on. From the fifteenth day, the nucleoli were red rather than a bluish-purple as in the unextracted series (Figs. 5 and 6).

Another series was extracted at 37° C for 24 hours. In this group, the metachromatic response of the nucleoli was considerably less than in the series extracted at room temperature. Here again the nucleoli stained metachromatically after 3 days of starvation. The size relationships of the nucleoli were essentially the same in extracted and unextracted nuclei. Interesting differences in other respects were noted. These included the tendency for the nucleoli to appear elongate, as well as pale and indistinct from the eleventh day of starvation.

These alterations were also observed in thin sections of starved amoebas treated with toluidine blue (as well as with Heidenhain's hematoxylin). In the sections, the nucleoli were enlarged (round or elongate) with or without paling, from the fourth day of starvation. On the fourteenth day (end of series B) the nucleoli appeared large and indistinct. This would seem to indicate that there may be a progressive increase in the water solubility of the material of the nucleoli during starvation. In order to demonstrate this in a whole mount, prolonged extraction was necessary. In a thin section

whose total volume is a small fraction of the whole mount, and whose exposed area is proportionately much larger, the very process of hydration and staining in an aqueous solution may be sufficient to bring about, within a short time, an extraction comparable to that brought about in whole mounts after 24 hours at 37° C.

Organic phosphates in normal amoebas. Organic phosphate, as indicated by the development of a blue color, was seen to be throughout the cytoplasm. The color was strongly concentrated in the nucleoli, in the food vacuoles, *i.e.*, in the food organisms within the vacuoles, and at the surfaces of vacuoles. The refractive bodies or their remnants which were seen only occasionally even in stratified preparations, appeared to be blue in color. It was initially thought that refractive bodies are probably dissolved by one of the reagents. The following evidence, however, indicates the paucity of refractive bodies to be due to other causes. Amoebas were incubated at 37° C in a dilute hydrolysis solution (*i.e.*, hydrochloric acid concentration reduced in half) and, after 24 hours, these amoebas were removed and treated with acetic benzidine and sodium acetate. The cytoplasm was observed to be unstained while the nucleus appeared bright blue. The intensely blue refractive bodies which appeared fused were seen in the periphery of the amoeba or adhering to the outer surface.

Sulfhydryl groups. The observations of sulfhydryl distribution which were made directly on living amoeba are in accord with the description given by Chalkley (7). The nucleus, especially the region of the nucleoli, becomes more intensely pink than the cytoplasm. Most food vacuoles show an intense coloration. The variations, as reported by Chalkley to exist between amoebas

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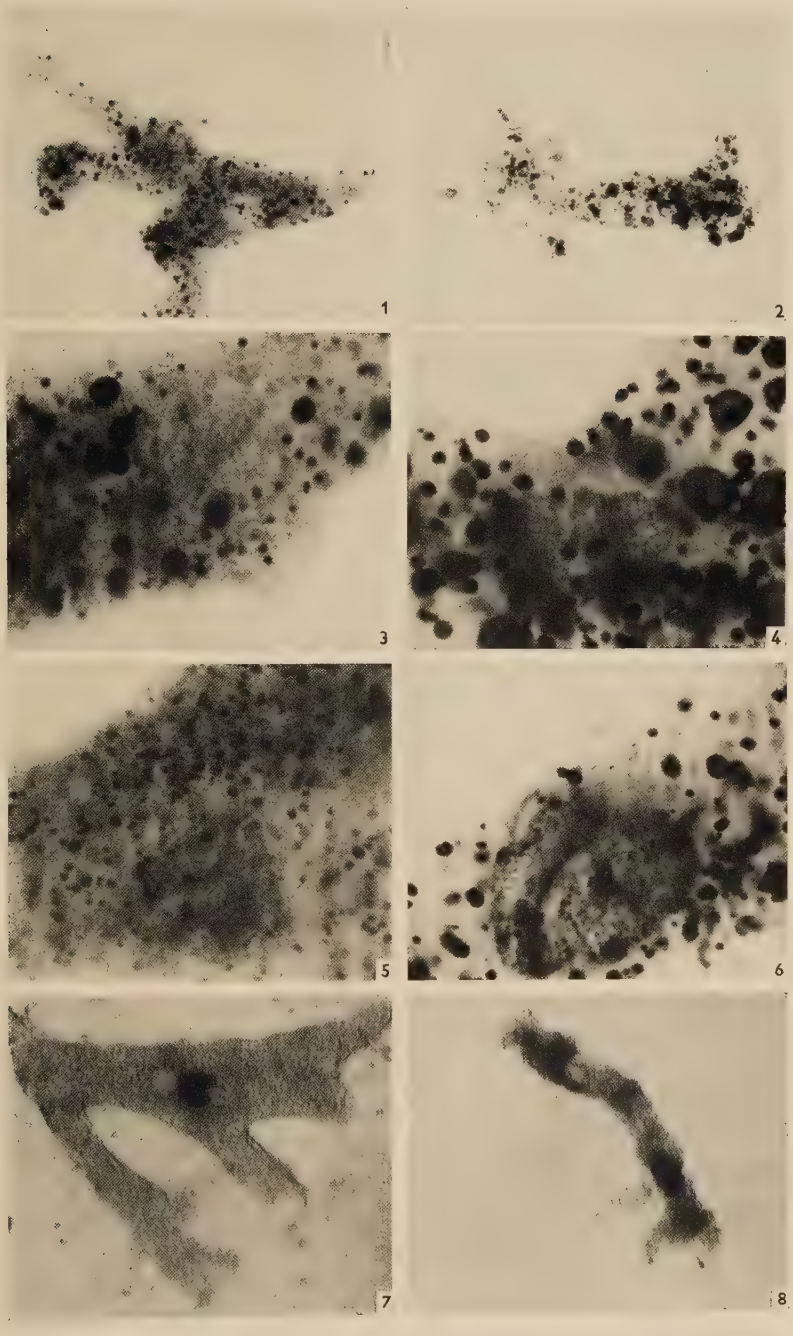
Figs. 1–2. Whole mount amoebas stained with toluidine blue. Photographed and printed under identical conditions. Cenco filter, no. 2. Approx. 200 ×. Fig. 1. One day of starvation. Fig. 2. Nineteen days of starvation. Note the striking reduction in the cytoplasmic coloration and the preponderance of very small refractive bodies. The darkly stained nucleus is in the right hand portion of the amoeba, surrounded by a number of enlarged refractive bodies.

Figs. 3 4. More highly magnified (approx. 1000 ×) portions of toluidine blue-stained, starved, whole mount amoebas. Cenco filter, no. 2. Fig. 3. One day of starvation. Note the pale nucleoli of the centrally located nucleus. Fig. 4. Nineteen days of starvation. Note the enlarged, more darkly stained nucleoli and the larger and darker refractive bodies. Photographed and printed under conditions identical to those used for Fig. 3.

Figs. 5 6. Whole mounts of starved amoebas stained with toluidine blue, after 24 hours of extraction in water. Approx. 1000 ×. Cenco filter, no. 2. Fig. 5. Three days of starvation. Note the pale nucleoli in the centrally located nucleus, and the tiny refractive bodies of the cytoplasm. Fig. 6. Nineteen days of starvation. Note the enlarged, more darkly stained nucleoli and the larger more intensely stained refractive bodies.

Figs. 7–8. Starved whole amoebas, fixed, treated with trichloracetic acid and with Chalkley's test for sulfhydryl groups. Photographed under identical conditions. Approx. 800 ×. Fig. 7. One day of starvation. Fig. 8. Fifteen days of starvation. Note the increased color intensity of the nucleus and the cytoplasm.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of different sizes, *i.e.*, less intense nuclear and more intense cytoplasmic reaction in younger as compared to older amoebas, were observed.

Amoebas (whole mounts and sections) when treated with sulfhydryl staining reagents after trichloroacetic acid treatment showed the same general distribution of sulfhydryl groups as was observed in living forms, but the intensity of the reaction was stronger.

The visualization of sulfhydryl groups in stratified amoebas treated with trichloroacetic acid definitely confirmed the observations which were made on unstratified amoebas. The rose color indicating sulfhydryl groups was distributed throughout the cytoplasm. The centrifugal region appeared darker by virtue of the presence of the deeply staining nucleus and food vacuoles, and a deeply staining cytoplasmic band or region, the depth of color depending upon the compactness of the strata. Application of the Regaud technique showed that this cytoplasmic band was rich in mitochondria. The lipid fraction concentrated at the centripetal end showed no color. The refractive bodies and crystals were determined to be uncolored.

Whole mounts which had been starved for one through 20 days were treated for 15 minutes with 10 per cent trichloroacetic acid prior to testing for sulfhydryl groups. Whole mounts of amoebas of the various days of starvation were treated simultaneously to insure uniform conditions. Color indicating sulfhydryl groups were seen in both the nucleus and cytoplasm of amoebas starved for 1 to 20 days. From one through three days of starvation amoebas appeared similar to the controls. From the fifth day through 9 to 13 days, when a maximum was reached, there appeared to be an increased intensity of color in the cytoplasm and even more so in the nucleus. The maximum color intensity obtained at about 13 days, was maintained through the twentieth day (Figs. 7 and 8).

DISCUSSION

The distribution of organic phosphate in the normal amoeba closely parallels the distribution of both free sulfhydryl groups (reduced glutathione, cysteine, and unbound sulfhydryl groups of proteins) and ribonucleic acid. All three substances have been detected in the cytoplasm and in the nucleoli. Sulfhydryl groups have been demonstrated in the mitochondria.

The association of these metabolically significant entities in the cytoplasm may be pertinent in protein synthesis. Holter and Kopac (12) found dipeptidase to be associated with the cytoplasmic matrix of *Amoeba proteus*. Hirshfield (personal communication) found cytochrome oxidase in the

cytoplasm of *Amoeba proteus*. He also found cytochrome reductase, although his method did not localize the site of the enzyme. Andresen, Engel and Holter (2) determined succinic dehydrogenase to be associated with the mitochondria of *Chaos chaos*. Holter and Løvtrup (13) associated proteinase activity with the mitochondria of *Chaos chaos*. These observations with those indicated in this paper, seem to suggest that the cytoplasmic matrix, the nucleoli, and the mitochondria may be the loci of protein synthesis in *Amoeba proteus*.

A progressive decrease of ribonucleic acid, indicated by staining with toluidine blue, which was observed in the cytoplasm of starving amoebas, paralleled an increase in both the size, and intensity of staining, of the nucleoli. Brachet (5) found little or no decrease in cytoplasmic ribonucleic acid as indicated by staining with pyronin, and noted a decrease in the number and basophilic reaction of the nucleoli at 10 to 12 days of starvation. The analyses which were carried out indicate that toluidine blue is specific and that pyronin B is non-specific for the detection of ribonucleic acid in the amoeba. This conclusion is in agreement with that of Kurnick (16) in respect to the action of these dyes. The lack of decrease in the pyronin-staining capacity of the cytoplasm of starving amoebas could be accounted for by an increase in non-specific reacting acidic groups, possibly in proteins, concomitant with the reduction in ribonucleic acid concentration. An alteration in protein reactivity has been observed, *i.e.* increased reactivity of protein-bound sulfhydryl in starving amoebas.

Part of the observed increased intensity of the toluidine blue staining of the nucleoli in starved amoebas is due to a metachromatic component. It was observed that as starvation progresses the metachromatic portion of the material of the nucleoli increases relative to the orthochromatic portion. Pyronin staining, therefore, might indicate a decrease in the concentration of the stainable material in the nucleoli.

The question arises as to the chemical nature of the chromotropic component of the material of the nucleoli. Lison (18) considered metachromatic staining to be due to half-esters of sulfuric acid with high molecular weight carbohydrates. Lison and Mustaars (20) have reported that nucleic acids may cause a metachromatic shift of the absorption curve of toluidine blue solutions *in vitro*. Flax and Himes (10) demonstrated metachromatic staining of ribonucleic acid by azure A in tissues. A progressive depolymerization of the ribonucleic acid with starvation might conceivably account for a shifting in its staining properties with toluidine blue.

In view of Holter and Zeuthen's (14) investigations, it seems highly

unlikely that the appreciable increase in the sulfhydryl content in both the cytoplasm and nucleus, during starvation, could be explained by an increase in the relative amount of protein. The increased sulfhydryl content might conceivably be due to: 1) a conversion during starvation of a progressively greater proportion of protein bisulfide to the reduced state, 2) a preferential utilization of protein low in sulfhydryl, 3) a progressive breakdown of lipids or carbohydrates or both, resulting in the unmasking of protein-bound sulfhydryl which were formerly undetectable by trichloracetic acid treatment, 4) a release of proteins from nucleo-protein complexes, by the breakdown of ribonucleic acids, or 5) a partial breakdown of protein molecules.

The data from the cytochemical localization of ribonucleic acids, desoxyribonucleic acids, and protein-thiols suggest that at least three key metabolic phases occur in starving amoebas, one from initiation to around the fifth day of starvation, a second from the fifth to the thirteenth day, and a third, from the thirteenth until the twentieth. In addition, there may be a fourth, the terminal metabolic phase, which occurs during cytolysis.

SUMMARY

The localization of desoxyribonucleic acids, ribonucleic acids, and sulfhydryl groups in normal and starving *Amoeba proteus* was investigated. In addition, the distribution of organic phosphates was determined in normal amoebas.

In normal amoebas, the cytoplasmic matrix and the karyosome were determined to contain relatively low concentrations of the following: organic phosphates, thiols, and ribonucleic acids. These substances, in higher concentration, are localized in the nucleoli. Desoxyribonucleic acids were indicated to be solely in the karyosomal portion of the nucleus. Within the cytoplasm, the mitochondria were found to contain thiols; the refractive bodies, a high concentration of phosphate and a component which stains metachromatically with toluidine blue; and the crystals, none of the substances tested.

During starvation, alterations were noted in the concentration and distribution of the compounds investigated. A progressive condensation of the Feulgen-positive karyosome was observed to occur between the fifth and thirteenth days of starvation, after which time the observed alterations were maintained until the termination of the experiment.

In the starved amoebas stained with toluidine blue there was observed a progressive reduction in cytoplasmic color from the fifth to the thirteenth day.

The nucleoli were observed to become progressively larger and slightly metachromatic, again leveling off at thirteen days. While considerable variation was noted in the size and metachromatic staining of the refractive bodies with toluidine blue staining throughout the series, the trend appeared to involve a darkening and reduction in size, as starvation progressed. The alterations of the refractive bodies suggest that some constituent may be removed during starvation.

The sulfhydryl content of the amoebas treated with trichloroacetic acid, appeared to increase in both the nucleus and cytoplasm during the fifth through the thirteenth day of starvation. The maximum content, reached at thirteen days, was maintained.

The major alterations determined in starving amoebas were progressive changes first noted on or about the fifth day of starvation, reaching a peak around the thirteenth day after which the level was maintained. The data establish that there are at least three key metabolic phases in *Amoeba proteus* during starvation.

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STUDIES ON GROWTH FACTORS FROM THE SEA URCHIN EGG

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Developmental physiology to a large extent deals with interactions between different parts of the germ, cf., the phenomena of physiological dominance, processes of induction, mutual adjustments in the size of organs (10), revitalization of genotypically lethal, heat-shocked, or lithium-checked blastema (2, 19), the Yamada effect (29), and the Rose effect (21). Interactions are of fundamental importance in the embryology of the sea urchin, *e.g.*, interactions between animal and vegetal parts of the early germ (11, 17, 22), and between the prospective organ rudiments in later development (9). These interactions may all involve the diffusion from one part of the embryo to another of tissue specific compounds (cf. 26), nutrients, intermediary metabolites, coenzymes, waste products, etc. It is likely that the excessive production or utilization of these compounds in one area of the germ interferes with the biochemical processes in the adjacent cells (6, 8). Areas of the embryo rich in such compounds may thus share their abundance with adjacent areas, (cf. 16). The interaction may also involve a competition between adjacent areas for these agents (cf. 27). A theoretical consideration of such interactions has been given (24).

Attempts have been made to elucidate the nature of the agents which mediate the interactions in the sea urchin germ. Straightforward extractions of animal and vegetal egg halves and a test of the animalizing or vegetalizing effects of these extracts have been made (12). The experimental conditions of such tests are very unfavorable, however, and the results have thus far been negative. A more stepwise approach to the problem was later made by systematic studies of the developmental changes in the embryonic content of various diffusible substances, such as amino acids, peptides, vitamins, and coenzymes, as well as the physiological effects of embryonic extracts on tissue culture cells and bacteria.

In a study of the amino acid composition on the developing sea urchin egg, great fluctuations in the peptide-containing fraction, *i.e.*, the non-protein, non-amino nitrogen fraction was demonstrated (7, Fig. 5). In a very syn-

chronous material these smooth curves may be resolved into several peaks (13), probably corresponding to a series of waves of differentiation in different areas, cf., the waves of mitochondrial development (9). The physiological activity of the peptide-containing fractions was demonstrated in microbiological experiments with lactic acid bacteria to which extracts from different stages of sea urchin embryos were added. Different bacteria responded differently. On a complete, synthetic medium, supplemented with a series of natural compounds, *Lactobacillus casei* showed a growth stimulation curve which greatly resembled that obtained by Van Slyke nitrogen determinations of the peptide-containing fraction (8, 14). On vitamin deficient media other curves or even similar curves were obtained. The growth promoting effect for *Lactobacillus casei* is not corresponding to the quantitative variations in the respective vitamins (results to be published from this laboratory), and is not eliminated by the addition of any vitamins. The nature of the growth promoting agents is discussed here in the light of chromatographic experiments. The use of *Lactobacillus casei* for these studies is justified on account of the great similarities between the nutritional requirements of this organism and animal cells. Thus, a certain unidentified growth promoting agent, the so called strepogenin, is active both for certain bacteria and rats (cf. 3).

Extractability of the growth promoting factors

The growth promoting agents in the sea urchin egg were assayed in alcohol (70 per cent) and tungstic acid extracts from various stages of development, and the activity was plotted against the stage of development (cf. (8), Figs. 2 and 5). In early stages of development the alcohol and tungstic acid curves are very similar in shape, both showing a decrease in the advanced blastula. After this stage, however, the curves diverge, i.e. the tungstic acid curve shows a rise, whereas the alcohol curve remains on a low level. It thus appears that the difference between the two curves reflects a developmental change in the relative amount of an alcohol soluble "blastula factor" and a non-alcohol soluble "gastrula factor". The former should decrease and the latter increase during the development.

An attempt was made to fractionate the growth promoting extracts utilizing the suggested difference in alcohol solubility. A gastrula water extract was used as a source of the factors. The extract was lyophilized, and the lyophilized residue was extracted first with 70 per cent alcohol and then with water. The extracts were then assayed for activity. Contrary to our expectation, all the activity was extracted with alcohol. This indicates that the dif-

ference between the "gastrula factor" and the "blastula factor" is a matter of extractability rather than solubility. Once the "gastrula factor" has been extracted with water it is soluble in alcohol.

Chromatographic studies

The studies above suggest that the main difference between early and late stages of development could be a matter of extractability of a single growth promoting substance. This factor could be progressively anchored, e.g., to the developing mitochondria in the gastrula and thereby made less extractable with alcohol. This question can only be settled by a study of the homogeneity of the extracts, e.g., by means of paper chromatography.

Paper chromatograms were run one-dimensionally with four different solvent systems, phenol, isobutyric acid, collidin, and pyridine-amylalcohol. The phenol was saturated with water, with traces of 8-hydroxyquinoline added, and used in the presence of ammonia in the gas phase. The isobutyric acid medium contained 25 parts isobutyric acid, 9 parts water, and one part concentrated ammonia. The collidine was saturated with water, and the pyridine-amylalcohol mixture contained 35 parts pyridine, 35 parts technical amyl alcohol, and 30 parts water according to the prescriptions of Edman (5).

The dried chromatograms were extracted repeatedly with boiling water in a Soxleth extraction apparatus, and the samples were evaporated to dryness on a boiling water bath in a current of air. The residue was taken up in water and added to the double concentrated media.

Paper chromatograms were run with alcohol extracts of young blastula stages. The tungstic acid extracts could be separated by means of phenol into at least three main components, one with a low R_f value, one with an intermediate, and one with a high value (Fig. 1 a). It is sometimes evident that one or two other components occur. In collidine the separation was poor; most of the components were overlapped near the starting point of the papers, but even here indications of the presence of more than one component were obtained (Fig. 1 b). Pyridine-amylalcohol gave essentially the same picture (Fig. 1 c). In isobutyric acid the components overlapped to a large extent in in the intermediate zone of the paper chromatogram (Fig. 1 d).

The chromatograms, especially those in phenol, proved that the extracts contained more than one growth promoting component. They further showed that there are developmental changes in the proportions between the different components which parallel the changes in the total activity. The rapid component in phenol thus appears to be relatively more abundant in ad-

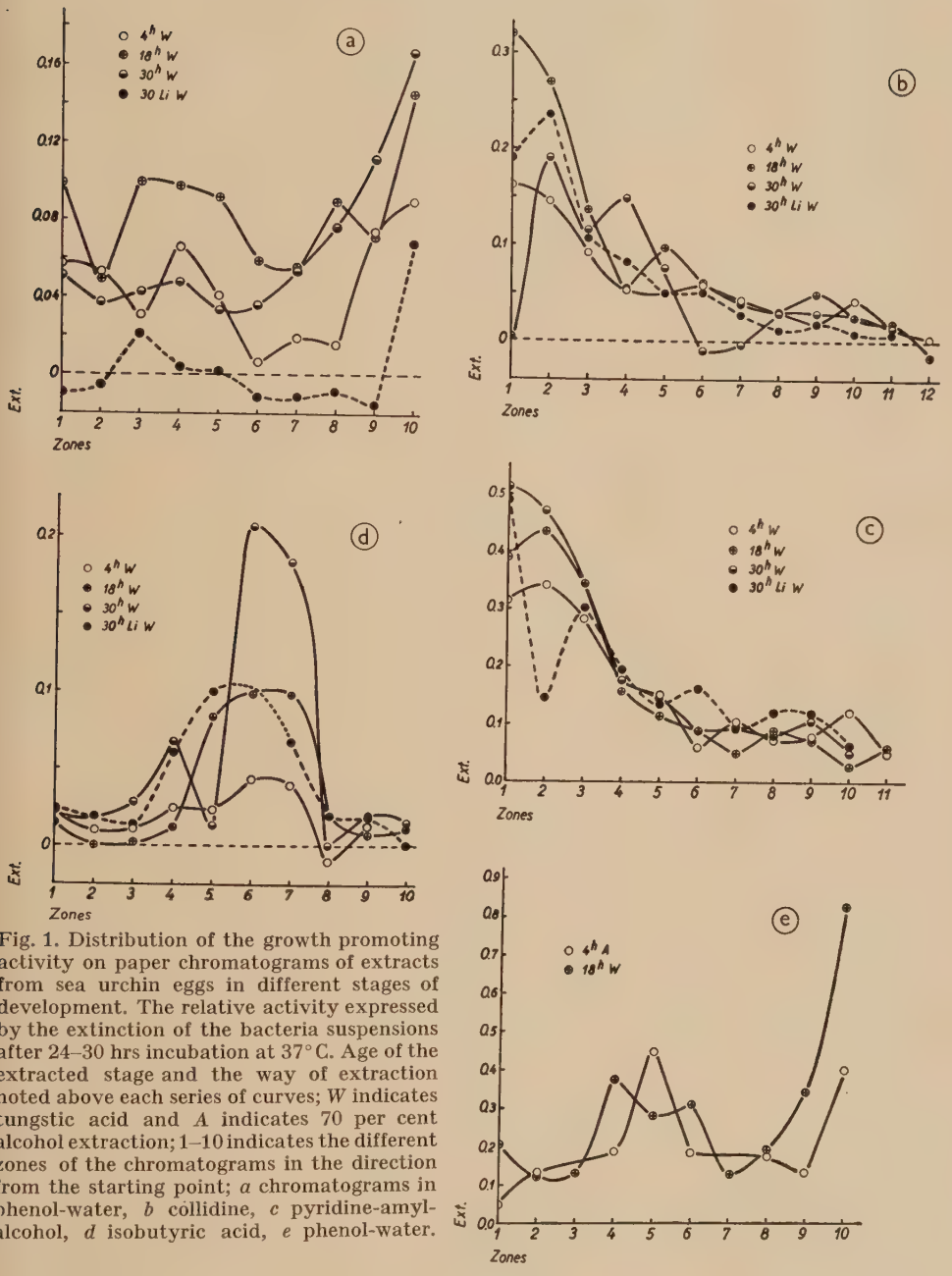


Fig. 1. Distribution of the growth promoting activity on paper chromatograms of extracts from sea urchin eggs in different stages of development. The relative activity expressed by the extinction of the bacteria suspensions after 24–30 hrs incubation at 37° C. Age of the extracted stage and the way of extraction noted above each series of curves; *W* indicates tungstic acid and *A* indicates 70 per cent alcohol extraction; 1–10 indicates the different zones of the chromatograms in the direction from the starting point; *a* chromatograms in phenol-water, *b* collidine, *c* pyridine-amyl-alcohol, *d* isobutyric acid, *e* phenol-water.

vanced stages of development. The result, however, indicate that the situation is not quite as simple as originally believed. The different factors do not appear to be restricted to earlier stages and gastrula stages respectively.

The phenol chromatograms of alcoholic and tungstic acid extracts of the 4-hour cleavage stage both show the presence of components with an intermediate and high R_f value. This indicates that the alcohol extraction is comparable with the tungstic acid extracts in the early cleavage stages. This was also indicated by the similar course of the developmental changes in growth promoting activity in these extracts (cf. (8), Figs. 2 and 3).

Strepogenin activity of the extracts and synergistic effects

The developmental changes in the growth promoting activity are similar to the changes in the peptide-containing fraction. This suggests that the growth promoting agents may be strepogenin active peptides, which are known to stimulate *Lactobacillus casei* under conditions similar to those used in our experiments (cf. 15, 25). If this suggestion is valid, the growth response to the embryonic extracts should be diminished after supplementing the basal medium with relatively high amounts of strepogenin. In order to elucidate this question, strepogenin prepared from insulin, kindly supplied by Dr. Kodicek, was added to the basal medium in various concentrations, and the growth stimulating activities of the young blastula alcohol extract and the late gastrula tungstic acid extract were studied. The blank without embryonic extracts showed a maximum stimulation at a strepogenin content corresponding to about 3 mg insulin per 5 ml final volume (Fig. 2). The response to the egg extracts was, unexpectedly, not diminished, but increased by the presence of strepogenin in the basal medium. This effect increased with the concentration of the strepogenin (Fig. 3). The effect of strepogenin on the response to the egg extracts is clear both in the case of alcoholic blastula extracts and tungstic acid gastrula extracts. Neither of the growth promoting extracts is thus identical in its action with strepogenin prepared from insulin by means of partial acid hydrolysis according to Kodicek (kindly supplied by Dr Kodicek).

The synergism between strepogenin prepared from insulin and the growth promoting activity of the embryonic extracts suggested that a synergism also might occur between the different chromatographic components of the embryonic extracts. If such a synergism occurs, new chromatographic compounds might be revealed if a small amount of unfractionated embryonic extract was added to the basal medium used for assaying the different zones

of the chromatograms for growth promoting activity. No additional components were, however, demonstrated in this way, but there was a great accentuation of certain peaks in the chromatograms (Fig. 4). There is thus some indication of a synergism between different components in the extracts.

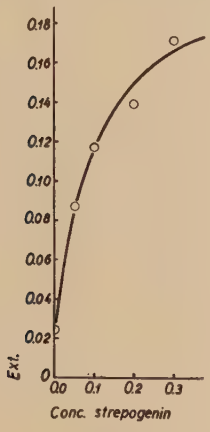


Fig. 2.

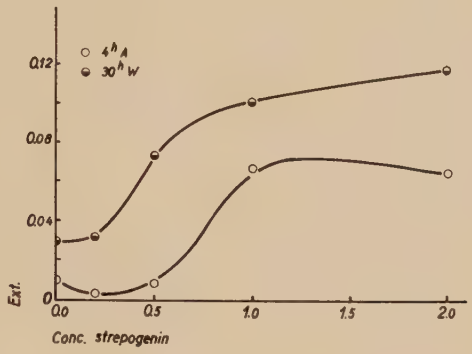


Fig. 3.

Fig. 2 (left). Growth response to strepogenin from partially acid hydrolyzed insulin prepared according to Kodicek. Conc. 0.1 denotes a strepogenin concentration corresponding to 1 mg of insulin per 5 ml medium.
Fig. 3 (right). Additional growth response to egg extracts in the presence of various amounts of strepogenin. (Growth in tubes with egg extracts plus strepogenin minus growth in tubes with strepogenin.)

The relation between the growth promoting agents and the peptide bond nitrogen

If the growth promoting agents are peptides, it might be possible to demonstrate a positive correlation between the zones on the chromatograms with a high peptide content and a high content of growth promoting substances. For that reason the Van Slyke amino nitrogen was determined before and after hydrolysis in different zones of the phenol chromatograms of embryonic extracts. The values were plotted against the R_f values and compared with the growth promoting activity of the different zones.

In one experiment a descending chromatogram on sheets of paper was run on tungstic acid extracts of 48-hour plutei (Fig. 5). An enormous peak of amino nitrogen, largely corresponding to glycine, dominated the diagram. The only significant change upon hydrolysis was a faint increase in a zone ahead of the glycine peak. The microbiological activity of the different zones was not determined in this experiment. There is, however, a certain positive

correlation between the peptide-containing level and the microbiologically active zone of a 30-hour pluteus-tungstic acid extract. The agreement is not complete but this may be partly accounted for by the differences in the chromatographic conditions in the different experiments. A complete compari-

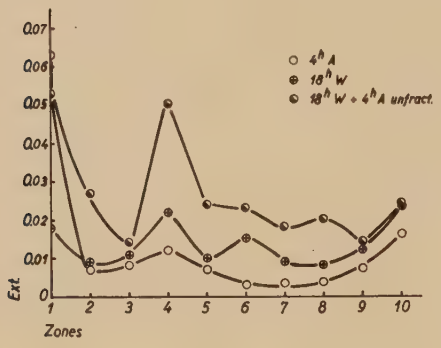


Fig. 4.

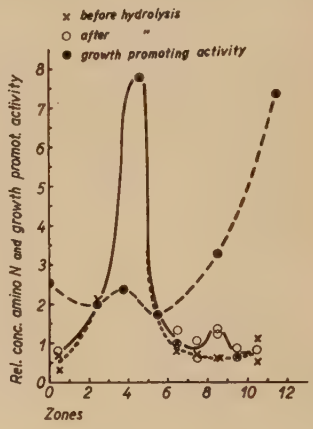


Fig. 5.

Fig. 4 (left). Distribution of the growth promoting activity on paper chromatograms of *a:* 70 per cent alcohol extracts of a 4 hr cleavage stage; *b:* tungstic acid extract of an 18 hr gastrula stage and *c:* the same stage as in *b*, but with medium supplemented with an unfractionated extract corresponding to a.

Fig. 5 (right). Relation between the Van Slyke amino nitrogen before and after hydrolysis and the growth promoting activity of a tungstic acid gastrula extract.

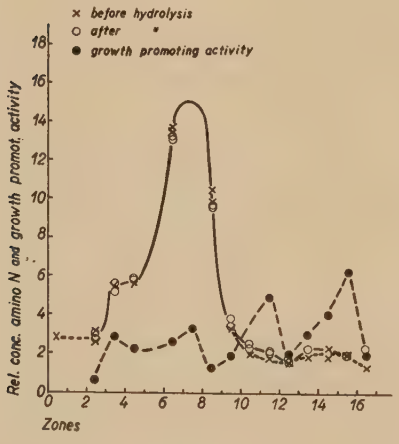


Fig. 6.

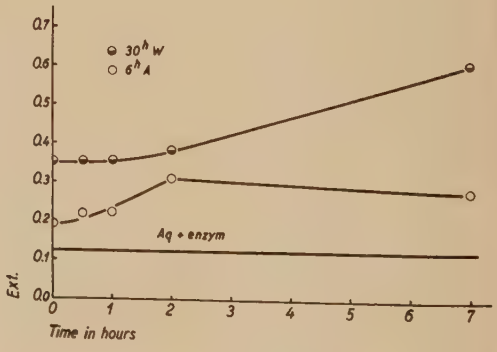


Fig. 7.

Fig. 6 (left). The same as 5 but extracts from a 4 hr abnormal cleavage stage.

Fig. 7 (right). Growth promoting activity of egg extracts treated with trypsin for 0–7 hrs.

.son can, unfortunately, not be made, as the points in zone 11 and 12 are lacking in the Van Slyke curve.

In a second experiment (Fig. 6), an ascending chromatogram with phenol was run on a roll of filter paper, this time on an extract of a 4-hour cleavage stage mixed with about 30 per cent abnormal cleavage stages. The Van Slyke determinations were paralleled by microbiological assays. The peptide values in this material are, however, very weak and dubious, and a comparison between the growth promoting zones is difficult.

An examination of the present results shows that the growth promoting activity calculated per peptide bond is very high and the activity is sometimes not clearly due to peptides. The question can only be settled after a study of much stronger extracts, from which the bulk of amino acids, at least the glycine, has been removed.

Stability of the growth promoting agents

The stability of the growth promoting agents was studied in order to get some indirect information about their chemical nature.

The activity of autoclaved extracts (two hours, 120° C) was compared with that of sterile filtered extracts. A 10 per cent decrease in activity was observed in the case of the tungstic acid gastrula extracts, whereas no destruction was noted in the alcoholic young blastula extract. Acid hydrolysis in 10 per cent HCl at 115° C for two hours destroyed about 98 per cent of the activity of the young blastula alcohol extracts and about 90 per cent of the activity of late gastrulae tungstic acid extracts.

The stability towards a series of proteolytic enzymes was also studied. The same blastula and gastrula extracts as above were used. *Trypsin and chymotrypsin* in a final concentration of 0.4 mg per ml were added to the egg extracts and the incubation at appropriate pH was continued for 7 hours at 37° C. Samples were removed immediately after the addition of the enzymes and after various times of incubation, and the enzymatic reaction was stopped by autoclaving the neutralized extracts. Chymotrypsin caused a very slight increase in activity. Trypsin caused an increase in activity which at least was very pronounced in the blastula alcohol extracts (Fig. 7). *Pepsin* used in the same way caused a 30 per cent destruction of the gastrula extract, asyptotically reached within half an hour. The inactivation of the blastula extract was of the same order but slower (Fig. 8). *Papain* prepared according to Balls and Lineweaver (1) and highly active against benzoyl-arginine amide caused a slight increase in activity of a tungstic acid pluteus extract in less

than an hour, whereas the young blastula alcohol extracts were unaffected. It is important to note that the enzymes themselves showed a strong growth promoting effect.

The results of these experiments indicate that the main growth promoting

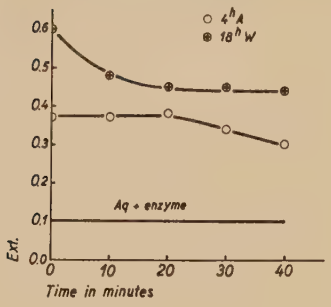
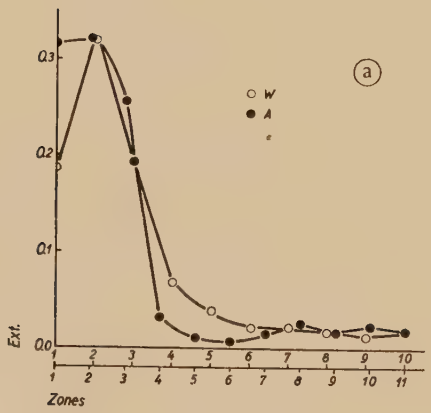
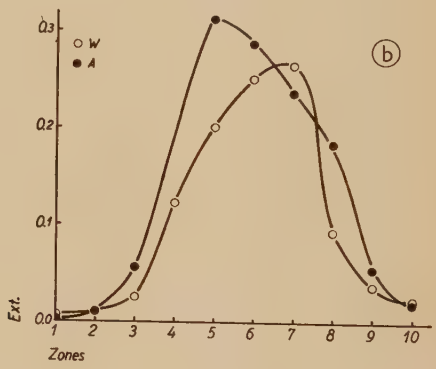


Fig. 8. The same as in 7 but pepsin treatment for 0–40 minutes.





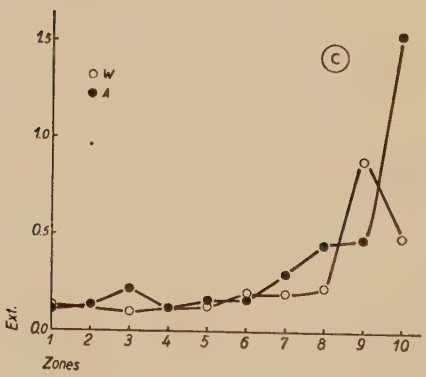


Fig. 9. Distribution of the growth promoting activity of Difco pepton extracts on paper chromatograms in various solvents. *W* Tungstic acid extracts, *A* 70 per cent alcohol extracts, *a* pyridine-amylalcohol, *b* isobutyric acid, *c* phenol-water.

effect is either due to non-peptidic components or to peptides very resistant towards proteolytic enzymes. It should be recalled in this connection that other strepogenin active agents are rather stable towards proteolytic enzymes, and they are even prepared by means of enzymic digestion of proteins (cf. 3, 28). This may explain why the activity of the extracts was sometimes increased by proteolytic digestion.

Chromatographic studies of growth promoting factors in other material

Various natural materials have been shown to contain growth promoting factors for *Lactobacillus casei*, increasing the growth of this organism on a synthetic medium (cf. e.g. 4, 15, 23). It was thus of interest to make a chromatographic comparison between these factors and those present in the egg extracts. Liver extracts (Park and Davis, "for intramuscular use"), strepogenin from insulin, and alcohol and tungstic acid extracts of Difco

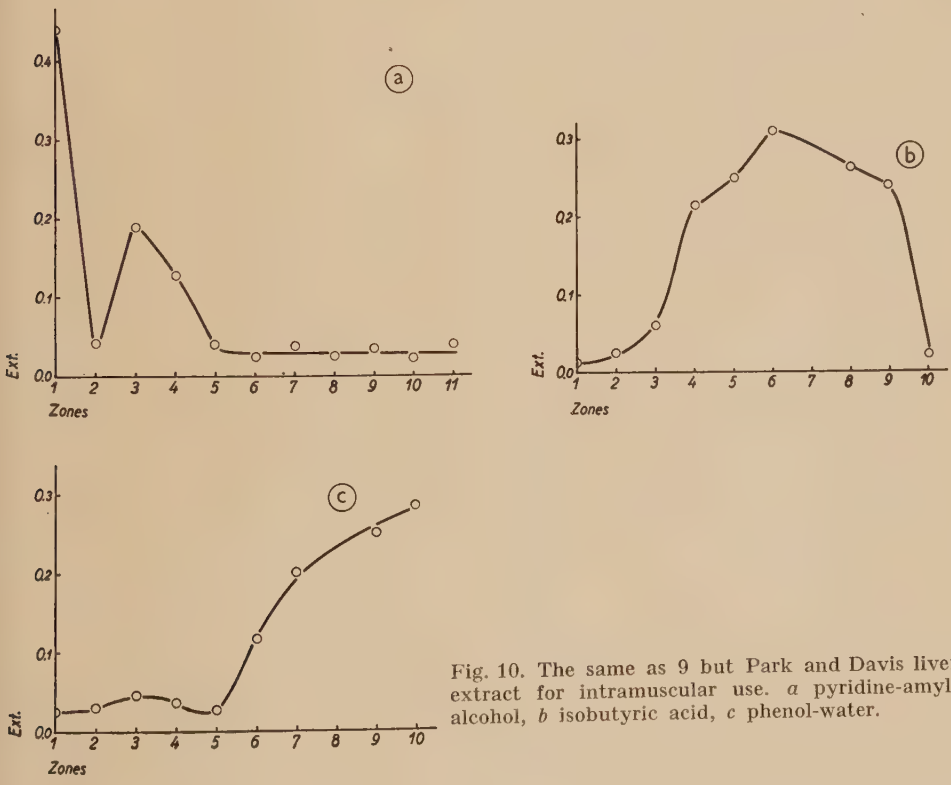


Fig. 10. The same as 9 but Park and Davis liver extract for intramuscular use. *a* pyridine-amyl-alcohol, *b* isobutyric acid, *c* phenol-water.

pepton were fractionated by means of paper chromatography with different solvents and the different zones were assayed microbiologically as above.

All the material tested showed growth promoting activity. The chromatograms often showed broad peaks of activity, but a further examination in-

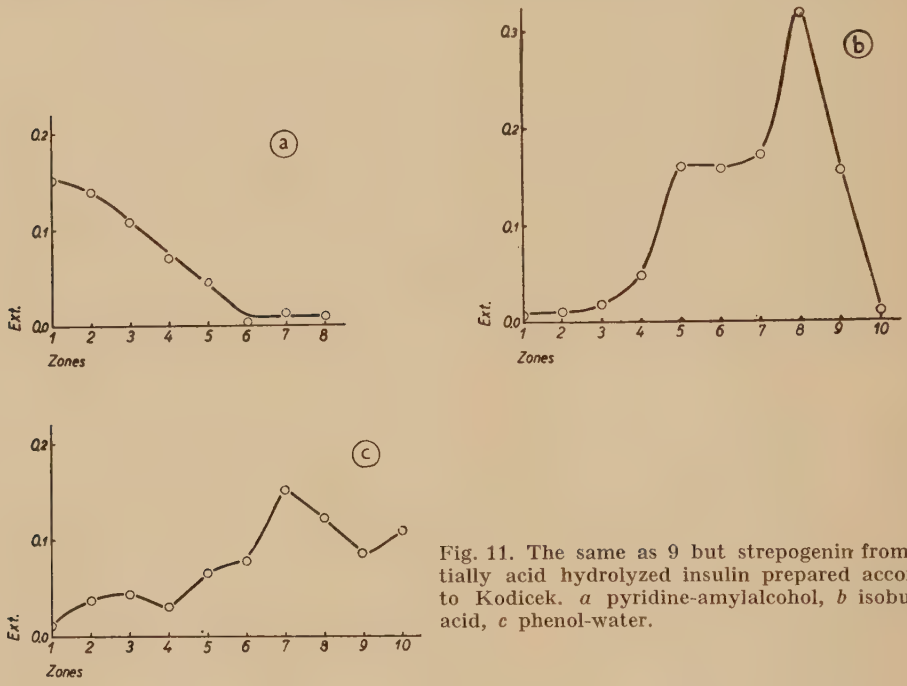


Fig. 11. The same as 9 but strepogenin from partially acid hydrolyzed insulin prepared according to Kodicek. *a* pyridine-amylalcohol, *b* isobutyric acid, *c* phenol-water.

dicated that these peaks are composite ones, as a rule, and consisting of several overlapping components. The general distribution of the activity showed striking similarities, i.e. there was a lower rate of migration in the basic solvents than in the acidic one and the migration was most rapid in phenol. There were, however, distinct differences, most apparent in phenol chromatograms where, e.g., the component with an intermediate R_f value is completely absent in pepton and absent or overlapped by a more rapid component in liver extracts (cf. Fig. 9–11).

Activity in liver homogenates and homogenized sea urchin eggs

It was supposed that if the growth promoting agents were peptides, they might be produced by transpeptidation reactions in homogenates in the presence of glutamine and glutathione.

Frog liver homogenates were incubated with glutamine and glutathione and amino acids. In certain experiments a strong growth promoting activity was demonstrated in the deproteinized filtrate, stronger in the presence of glutamine and glutathione than without. However, the activity decreased again after prolonged incubation. These experiments were repeated with homogenates of different developmental stages of sea urchin eggs. In this case the incubation medium was a complete medium for microbiological assay, supplemented with glutathione to a final concentration of 0.01 M, or without glutathione. Aliquots of the autoclaved and centrifuged samples were diluted with normal medium and assayed. The results were not consistent, but even in these experiments an increase and a subsequent decrease in activity could be noted. These homogenate experiments were considered to be too complicated, but it is evident that growth promoting compounds can be formed or released and then finally destroyed in homogenates.

DISCUSSION

The growth promotion of *Lactobacillus casei* by sea urchin egg extract is evidently due to a complex of factors. There is a great deal of evidence that the factors are related to strepogenin. The basal medium was thus adjusted (8) for strepogenin assay with *Lactobacillus casei*, and the factors were found to decrease the length of the lag phase, as does strepogenin. There is also a clear similarity between the chromatographic properties of the activity in the sea urchin extracts and that of various other natural materials with a documented strepogenin activity; the activity migrates as if it were associated with acid components with a high rate of migration in phenol. The low extractability of certain fractions of the activity in advanced stages of development may be of significance in connection with the fact that strepogenin is unextractable in 70 per cent alcohol, according to Woolley's original definition of strepogenin (25). Finally, there is a certain degree of correlation between growth stimulation and the peptide-containing zone on the chromatogram of a pluteus extract. The stability of the activity to proteolytic enzymes does not necessarily deny that the active molecules are peptides, since strepogenin is also rather stable under these conditions.

The strepogenin activity of the extracts of sea urchin embryos appears to be the first to be described for a natural system. Other known strepogenins are obtained from more or less degradated materials, e.g., acid treated insulin and trypsin digested or more or less autolyzed material. The naturally occurring strepogenins from the sea urchin larvae are, however, not equivalent

with Kodicek's strepogenin from acid treated insulin, one of the richest sources of this factor. There is a synergistic effect between egg extracts and the actual strepogenin preparation from insulin. Furthermore, some of the activity in the sea urchin is extractable with 70 per cent alcohol, whereas strepogenin in the restricted sense should not be.

The difference between the strepogenin activity in the sea urchin egg and that from insulin may indicate that the activity of the embryonic extracts is due to both strepogenin in the restricted sense and an additional factor or factors with a synergistic effect. This idea is in line with several observations which show that for optimal early growth *Lactobacillus casei* needs, besides strepogenin in the restricted sense, some alcohol-soluble factor of unknown nature which is present in liver extracts and various other material of animal origin (4, 23). It is also in line with the observations that a synergism appears to occur between certain fractions in the phenol chromatograms and factors in an unfractionated extract. It is, however, not possible to ascribe the true strepogenin effect to the rapid component and the complementary effect to the slower one, or vice versa.

The developmental changes in the relative amounts of the different components is evident from Fig. 1 a. The activity associated with the slow (R_f 0.5) component of the phenol chromatograms thus decreases during development, whereas that associated with the rapid component increases. The change in the relative abundance of the two different growth promoting factors was already suggested in our previous paper, where it was stated that the blastula stages were mainly characterized by an alcohol soluble blastula factor, and the more advanced stages of development by a non-alcohol soluble gastrula factor. One should, however, not speak about these factors as synonyms for the slow and the rapid chromatographic components, respectively, in phenol. There is thus only a gradual change in the relative proportions of the two components during development, and no abrupt one at the transition from the late blastula to the early gastrula. Furthermore, alcohol extracts from early stages of development contain both the slow and the rapid component, although relatively more of the former. Thus the apparent decrease in the alcohol soluble agents does not necessarily reflect a decrease in a distinct compound, but might even be caused by anchoring of various growth promoting agents to certain cellular structures. In this way they might become less extractable with 70 per cent alcohol. This interpretation is in accord with the extractability studies described above. It is possible that this anchoring occurs primarily in the mitochondria for those are known to develop in the advanced blastula and gastrula (9). An analogous

phenomenon seems to occur with cozymase in the amphibian development (cf. 18). Here the content of extractable cozymase drops sharply in the neurula, i.e., at a stage where the differentiation becomes intensified.

The role of the strepogenin active factors development of the sea urchin egg is unknown. There is some evidence (8, 14) that they play an important role in protein synthesis and in the biochemical interactions between different parts of the germ. The treatment of isolated animal and vegetal egg halves with different purified strepogenin fractions might be a logical extension of the present work. The effect of these fractions on tissue culture cells is now being studied.

SUMMARY

Developing sea urchin eggs contain factors extractable with water, tungstic acid, and 70 per cent alcohol, which have a growth promoting effect for *Lactobacillus casei* grown on a complete synthetic medium.

The growth promoting activity is due to at least two distinct chromatographic components.

The relative amount of the two components changes during development, and a change also occurs in the extractability of the factors.

The biological, chromatographic, and chemical properties of the active components favour the view that these naturally occurring substances are related to strepogenin. However, the activity of the extracts is not identical with that of strepogenin itself. There is evidence that the sea urchin egg also contains growth promoting factors which act synergistically with strepogenin prepared from partially acid-hydrolyzed insulin.

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STUDIES ON ISOLATED CELL COMPONENTS

VII. A REEXAMINATION OF THE PREPARATION AND PROPERTIES OF RAT LIVER HOMOGENATES[1]

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The techniques of differential centrifugation of tissue homogenates, introduced by Bensley and Hoerr (4), and developed by Claude (6), Hogeboom *et al.* (12) and many others, has found wide application in the study of enzymes and enzyme systems, in the investigation of certain physiological properties of various cell components, and in the initial stages of the purification of nucleic acids and various proteins. The methods appear for the most part to have been empirically devised and have been found to result in a degree of separation which does not approach that which would be expected from purely theoretical considerations. It has been considered desirable therefore to reexamine a number of aspects of these techniques. This paper will be concerned with the preparation and properties of rat liver homogenates; a subsequent paper will deal with the techniques of high resolution differential centrifugation.

HOMOGENATE PREPARATIONS

Tissue homogenates, such as those used in the present series of studies for the preparation of isolated nuclei, may be considered as true solutions containing a number of micro-ions, as colloidal suspensions containing particles covering the entire range of sizes customarily ascribed to colloids, and as slurries with particles large enough to settle out in the course of a few hours. In such a complex system a number of factors may be expected to have marked effects. These include the pH, density, viscosity, and composition of the suspending medium; the size distribution, density, and colloidal properties of the particulates, and the method and solutions used for tissue perfusion. Furthermore, it is not possible to eliminate completely

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the rapid changes which occur during and after cell death. It is important to determine as far as possible whether homogenization involves the rapid rupture of healthy cells, or the maceration of injured, cytolyzing ones—in short, whether or not the technique is really an exercise in necrobiology.

EXPERIMENTAL

Perfusion.—In the nuclear isolation method previously described (20) livers were perfused via the dorsal aorta using cold 0.15 *M* NaCl. Recently the use of cold sucrose-$CaCl_2$ solution introduced through the hepatic portal vein has been advocated (13). Other workers have generally used either saline or a balanced salt mixture such as Locke's solution. The dilemma at this point is whether to be concerned with the state of intactness of the cells or with changes occurring in cell components after cell breakage in unsuitable solutions. The amount of perfusion fluid remaining in the liver is probably around 20 g per 100 g of liver since the mass of extracellular material in the rat liver is about 24 g per 100 g of tissue (10). In mice, the values may be expected to be somewhat higher since the volume occupied by blood is 27 per cent of the liver volume for males and 31 per cent for females (17). When a 10 per cent homogenate is prepared, the concentration of the perfusion medium will be 2–3 per cent. It has been considered of interest therefore to study the effects of different solutions on cell breakage and on the amount of residual stroma.

Perfusion technique.—The following method has been used for some time and has been found to provide essentially blood-free livers in a very short space of time.

The rat is stunned or killed by cervical luxation, rapidly decapitated, and allowed to bleed for a few seconds while suspended by the tail. The skin covering the entire left side of the body is quickly removed using heavy curved stainless steel autopsy scissors. An incision is made on the left side below the ribs, continued around to the spine, and along the spinal column to the top of the pleural cavity severing all ribs in between. The diaphragm is cut close to the chest wall around to the midline. The aorta is then cut halfway through about $1\,^1/_2$ cm above the diaphragm, leaving a flap pointing anteriorly. A thin-walled glass cannula is then inserted with the perfusion fluid streaming out, thus washing away surrounding blood during insertion. The perfusion fluid is maintained under a pressure of 120 cm of water to approximate the normal rat blood pressure (9). The left lobe of the lung is then removed to allow free outflow of blood. The inferior vena cava is also nicked. The vagus nerve is generally severed during this procedure, but this has been shown to have no obvious effects on liver circulation (18). Perfusion is generally completed within 1–2 minutes, and is continued while the liver is being removed.

For complete perfusion it has been found advisable not to chill the liver until after perfusion has been started. Cold Locke's solution is poured over the liver as soon as it begins to clear. The success of perfusion through the aorta, in view of the double circulation of the liver, is evidently due to the fact that the perfusion solution flows in through the hepatic artery and out both the hepatic vein and the hepatic portal vein, since the posterior half of the animal contains considerable blood when the liver is blood free. In some instances it has been found advantageous to cut the hepatic portal vein near the end of perfusion.

Perfusion is thus begun 30–50 seconds after death, the liver is cooled a minute or so later, and removed for weighing by the end of about 4 minutes. Every effort is made to minimize the time between death and the completion of perfusion. That rapid changes may occur is shown by the finding that a 15-minute interruption of liver circulation in the dog generally proves fatal (19), while in the rat kidney enspherulation and fragmentation of mitochondria occur in a few proximal tubules within 6 minutes after closure of the renal artery (8). The perfused livers are weighed in tared beakers of cold Locke's solution, blotted, and transferred to a cold graduate cylinder where sufficient homogenization medium is added to give the desired final homogenate volume.

If perfusion is omitted, a number of aggregates of cytoplasmic particulates are seen which sediment with the nuclei. These are probably bound together by fibrin. Perfusion therefore appears essential.

Effects of perfusion with various solutions.—The central role of divalent cations in the surface precipitation reaction (11) suggests that homogenization in the presence of substances such as citrate which bind calcium might result in better homogenization and fewer partially broken cells. Dounce (7) has suggested that this may be one of the reasons for the success of his citric acid method. To study this possibility livers were perfused with solutions containing 1 part of calcium-free Locke's solution and 1 part of isotonic sodium citrate. Homogenates were then prepared in Solution I (20) and were found to consist chiefly of microscopically intact whole cells. Similar results were obtained with sodium pyrophosphate, adenosinetriphosphate, and ethylenediamine tetraacetate. A method for preparing suspensions of whole cells based on these observations has been reported elsewhere (2). The concentration of calcium-binding substances may be reduced to about 0.01 *M* and still be effective. It is evident that under these conditions the removal of calcium softens the intercellular cement with results identical with those long known in similarly treated embryos (2).

In view of these results it was considered of interest to study the effects of perfusion with Locke's solution, both with and without calcium, and with sucrose containing calcium (0.25 *M* sucrose + 0.0018 *M* $CaCl_2$), and sucrose alone (0.25 *M*). Perfusion was accomplished as described and weighing was done in a cold beaker of the perfusion medium. Livers perfused with Locke's solution were homogenized in Solution I, while those perfused with sucrose solutions were homogenized in the perfusion solution. All homogenates were prepared in the same homogenizer (1.005 inches inside diameter, 10 inches long, with a plastic pestle 0.986 inches in diameter), and were given 70 even up-and-down strokes by hand with the homogenizer set in ice. Fifty to sixty milliliters of 10 per cent homogenate was prepared from each animal and strained through 12 ×× bolting silk.

As will be shown in a subsequent communication, a number of centrifugation artefacts exist which prevent cell components from sedimenting ideally. For this reason, the amount of material which might be expected to sediment with or ahead of liver nuclei (here referred to as contamination) was estimated by making direct counts with the phase contrast microscope rather than by centrifugal techniques. Homogenates were diluted with three volumes of the suspending medium. An average of 800 nuclei on five slides was counted from each preparation. The numbers of free nuclei (N), whole cells (WC), and broken cells (BC) or particles of debris which might sediment with the nuclei were tabulated separately. The number of free nuclei plus the number of whole cells gave the total number of nuclei present. Each cell fragment or particle of debris was considered to be the equivalent of one nucleus, while the amount of cytoplasm present in each whole cell was considered to be the equivalent of seven nuclei. The contamination present was considered to be equal to the sum, (WC × 7) + BC, and the percentage contamination was found from the equation:

$$\frac{(WC \times 7) + BC}{N + WC + (WC \times 7) + BC} = \text{percentage contamination.}$$

This method of calculation involves several simplifying assumptions, since the presence of about 31 per cent of binucleate cells (16) is disregarded, and all broken cells and debris particles are obviously not the same size as nuclei. However it is considered useful for comparative purposes.

The results obtained with sixteen male Sprague Dawley rats (213–270 g) are shown in Table I. The findings with Locke's solution both with and without calcium, and with sucrose plus calcium are similar, while a definite decrease in the amount of contamination is seen with 0.25 *M* sucrose.

TABLE I

Effect of perfusion with various solutions on the amount of material present which may sediment with nuclei and on the number of whole cells present.

Complete Locke's		Ca-Free Locke's		0.25 *M* Sucrose 0.0018 *M* $CaCl_2$		0.25 *M* Sucrose	
% Contam.[1]	WC/100 N[2]	% Contam.[1]	WC/100 N[2]	% Contam.[1]	WC/100 N[2]	% Contam.[1]	WC/100 N[2]
30.3	2.5	24.1	2.6	29.1	3.5	21.6	2.7
29.0	2.4	29.1	3.0	22.5	1.6	13.4	1.7
20.9	0.6	37.1	5.7	23.6	2.1	13.4	1.9
25.2	1.8	29.3	2.1	19.7	1.6	7.4	1.0
Average:							
26.3	1.8	29.9	3.3	23.7	2.2	13.9	1.8

The effect of pH on homogenization.—It has been suggested that best cell breakage is obtained at an alkaline pH (4). Dounce (7) found that nuclei disappeared when the pH was raised to 6.5 and suggested that this may be due to the action of cytoplasmic enzymes. Since no salts were used it is evident that, as pH 7.0 is approached with citric acid, the medium becomes essentially like distilled water which has been shown to cause marked swelling and disruption of fresh nuclei (3). The effect of pH on homogenization appeared therefore to merit further investigation.

Two complete sets of experiments were performed, the first with Osborne-Mendel, the second with Sprague-Dawley strain rats. Since the results are almost identical, only the findings with the latter strain will be reported. The livers of nine male rats (249–304 g) were perfused as described with complete Locke's solution. These were homogenized (70 strokes with the homogenizer described in the preceding section) in solutions of varying pH made by mixing different proportions of a solution containing 0.023 *M* KH_2PO_4 and 0.145 *M* sucrose with a solution containing 0.023 *M* K_2HPO_4 and 0.145 *M* sucrose. The lowest and highest pH's were achieved by using one or the other of these solutions alone. The solutions resemble Solution I in total salt concentration and in sucrose content. All homogenates were prepared in such a manner as to contain 1 g of liver per 10 ml of homogenate, and were strained through bolting silk.

The results are shown in Fig. 1 where contamination, calculated as described, is plotted against homogenate pH. It is evident that a sharp decrease

[1] Percentage calculated as described in text.
[2] WC/100 N = Number of whole cells counted per 100 nuclei.

in the amount of contamination occurs as the pH is raised from 5.8 to 7.8. This was found to be due to a decrease in the number of whole cells at the higher pH's and also to the fact that the cell wall material is free of attached cytoplasm at pH 7.8, and consists of very small shreds of material which probably would not sediment with nuclei. In the lower pH range the cell-wall material is generally fairly thick and often has attached coagulated

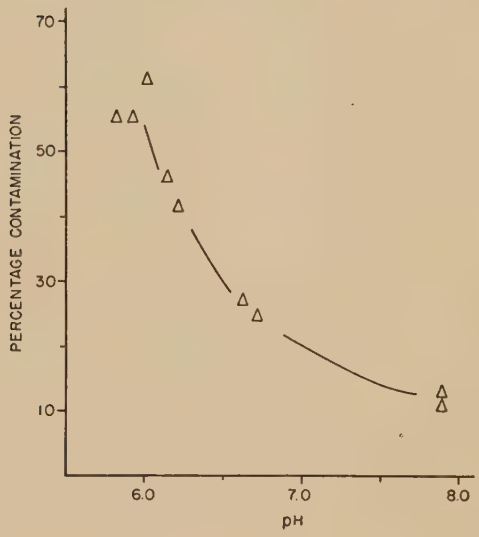

Fig. 1. Percentage contamination found in rat liver homogenates prepared in 0.023 *M* potassium phosphate buffers containing 0.145 *M* sucrose. The technique for preparing homogenates and the method for calculating contamination are described in the text.

cytoplasm. Very few half-broken (and therefore coagulated) cells were seen at pH 7.8. It is evident that under these conditions optimal cell breakage and dispersion of the cytoplasm occurs at a pH above 7.0.

Density of rat liver.—Relative density measurements were made on three livers from 2-month-old female rats of the Sprague-Dawley strain. These were rapidly perfused with Locke's solution and then weighed in air and in 0.28 *M* NaCl. The data of Opie (15) indicate that rat liver does not gain or lose water in this solution. The values obtained were 1.084, 1.085, and 1.088 g per cc with an average of 1.086. All measurements were made at 20° C. These values are in excellent agreement with those reported by Mori and Momoki (14) which were 1.088 for male and 1.084 for female rat livers. Perfused rat liver therefore has approximately the same density as 0.62 *M* sucrose. Homogenates prepared in more concentrated sucrose solutions may be layered over the homogenization medium since its density will be greater than that of the homogenate. Conversely, homogenates prepared in

TABLE II

Viscosities and densities of various solutions used for nuclear isolation. Densities are expressed in grams per cubic centimeter *in vacuo* at 0° C of solutions made up in double distilled water at 20°. Viscosities are calculated from the data of Bingham and Jackson (5). Addition of indicated salts to various sucrose solutions was found to have no detectable effect on viscosities.

Solution			Viscosity Centipoises at indicated temperatures			Density g/cc at 0° C
Description	Molarity of sucrose	Sucrose g/liter	0°	5°	10°	
3 × isotonic	0.88	301.2	5.75	4.61	3.80	1.1185 (1.1207[2])
"isotonic"	0.25	85.57	2.34	1.97	1.68	1.0338
Solution I[1]	0.145[2]	49.6	2.07	1.75	1.50	1.0224
Solution II[1]	0.218[2]	74.6	2.25	1.90	1.63	1.0323
Solution III[1]	0.272[2]	93.1	2.39	2.02	1.72	1.0395
Used in layering experiments	0.190[2]	65.0	2.18	1.85	1.58	1.0285
,,	0.219[2]	75.0	2.26	1.90	1.63	1.0326
,,	0.234[2]	80.0	2.29	1.93	1.65	1.0345[3]
,,	0.248[2]	85.0	2.33	1.96	1.68	1.0364[3]
,,	0.263[2]	90.0	2.38	2.00	1.71	1.0385
,,	0.584[2]	200	3.57	2.98	2.50	1.0819
,,	1.169[2]	400	10.0	7.58	6.25	1.1593
,,	1.753[2]	600	41.7	26.3	20.4	1.2350
Distilled water	—	—	1.7921	1.5188	1.3077	0.9998

solutions of lower concentration can be layered only over a solution containing a higher concentration of sucrose.

Physical properties of various media.—In order to calculate the theoretical sedimentation rates of various cell components it is necessary to know their size and density, and the density and viscosity of the solutions used. The viscosity of several sucrose-containing solutions used in studying isolated cell components has been calculated from the data of Bingham and Jackson (5) and the results are presented in Table II. It has been determined experimentally that the addition of small amount of salts (0.0125 *M* K_2HPO_4, 0.0094 *M* KH_2PO_4, and 0.0015 *M* $NaHCO_3$) to sucrose solutions over the range 0.145–0.88 *M* does not alter the viscosity detectably. The absolute densities of the same series of solutions have been experimentally determined

[1] Solutions used in nuclear isolation procedure previously described by Wilbur and Anderson (20).

[2] These solutions contain in addition to the sucrose concentration shown the following salts: 0.0125 *M* K_2HPO_4, 0.0094 *M* KH_2PO_4, 0.0015 *M* $NaHCO_3$.

[3] By interpolation.

at 0° C by use of a specially designed 100-ml pycnometer which could be filled accurately at 0° C and weighed after warming to room temperature. All weighings were corrected to *in vacuo* conditions and are expressed in terms of grams per cubic centimeter. Determinations were made in triplicate. These results are included in Table II. Solution viscosities are given at several temperatures since the values diminish rapidly with rising temperature

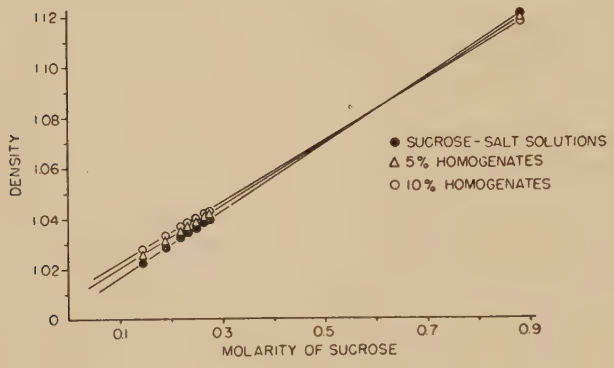

Fig. 2. Density of solutions containing 0.0125 *M* K_2HPO_4, 0.0094 *M* KH_2PO_4, 0.0015 *M* $NaHCO_3$, and concentrations of sucrose as indicated. Curves with open symbols show calculated densities of 10 and 5 per cent homogenates prepared in the same solutions. Since all solutions contain the same amount of salt, and liver tissue is present in preparations represented by two of the curves, none of the curves will intercept the zero point. All data for 0° C.

for any given solution. The densities, however, are given for 0° C only since little change occurs over the range 0–10° because the density of water passes through a maximum at 4° and is the same at 8.1° as at 0.0°.

Requirements for layering homogenates.—Assuming a density of 1.086 for rat liver, the densities of homogenates containing 1 g and 0.5 g of liver for each 10 ml of homogenate may be readily calculated, and are shown in Fig. 2. From these curves the density of a sucrose solution required to support a layer of homogenate made in any other salt-sucrose solution may be readily read. A salt-sucrose solution having the same density as a homogenate made in Solution I would have to have a density greater than Solution I by a value of at least 0.0045, while a similar solution having the same density as a homogenate prepared in Solution III would have to have a density greater by 0.0035. In the neighborhood of a sucrose molarity of 0.62 the salt-sucrose solution would have the same density as the solution used in making it. These values set the general limits of any layering procedure which employs sucrose, and allow a system to be set up at whatever sucrose concentration may appear desirable. As will be shown in a subsequent paper, simple layering procedures are subject to several artefacts which are more evident when the density differences are low.

Colloidal properties of homogenates.—The sign of the charge on proto-

plasmic colloids has been the subject of considerable disagreement (11). The following observations, however, support the conclusion that the colloidal constituents of a homogenate bear a *negative* charge. Addition of very small amounts of protamine, Janus green B, or other polycations has been found to flocculate homogenates and to cause rapid sedimentation of all homogenate constituents in very low centrifugal fields (1). The addition of strong polyanions such as heparin, ATP, or polysulfonated dyes (Erie fast rubine, solantine red, wool red 40F, brilliant scarlet 3R) produces no comparable effects. The gradual flocculation of homogenates observed on standing may well be due to depolymerization of nucleic acids and a destruction of ATP, resulting in a decrease in colloid associated negative charges. It is not unlikely that similar changes occur in cytolyzing cells.

DISCUSSION

The results presented allow a more rational approach to the problem of the preparation and fractionation of tissue homogenates. The inclusion of calcium-binding substances in the perfusion solution increases markedly the number of whole cells appearing in the finished brei. No appreciable difference was found, however, when cell breakage after perfusion with both complete and calcium-free Locke's solution were compared. When 0.25 *M* sucrose was used, addition of 0.0018 *M* $CaCl_2$ actually increased the amount of contamination observed.

Variations in the pH of the homogenization medium were found to have a marked effect on the composition of the homogenate. At a slightly acid pH many whole cells, partially disrupted cells, and cell walls with associated coagulated cytoplasm were seen. In contrast, above pH 7 few whole cells and almost no broken cells were found, whereas the intercellular material was represented by small shreds with no adherent cytoplasm. These findings suggest that homogenization is best accomplished above pH 7. The application of the data presented on the physical properties of homogenates and suspending media to the development of high resolution differential centrifugation is described in a subsequent communication.

SUMMARY

Several aspects of the preparation of homogenates and certain physical properties of suspending media have been examined in an attempt to develop a more rational approach to the problem of the differential centrifugation

of tissue homogenates. The addition of calcium-binding agents to the perfusion solution has been found to result in the presence of a large number of whole cells in liver breis. No difference in cell breakage was observed when calcium-free Locke's solution was compared with complete Locke's solution. The addition of 0.0018 *M* $CaCl_2$ to a perfusion medium consisting of 0.25 *M* sucrose resulted in an increase in the number of whole cells and cell wall material when compared with sucrose alone.

Comparison of the composition of homogenates identically prepared except for variations in the pH of the homogenization medium showed that at pH 6 a large number of whole cells, broken cells, and cell wall fragments were present. The number of these contaminants decreased markedly as the pH was raised to 7.88.

Data on the viscosity and density of a number of suspending media, on the density of perfused liver, and on the density of homogenates are presented. From these the requirements for the preparation of layered systems for differential centrifugation can be calculated.

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THE NUCLEIC ACID CONTENT OF CELLS IN THE MERISTEMATIC ELONGATING AND FULLY ELONGATED SEGMENTS OF ROOTS OF *VICIA FABA*

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In connection with studies of the rate of incorporation of phosphorus into the desoxyribose nucleic acid of meristematic cells of *Vicia faba* roots (8, 9), an attempt has been made to estimate in absolute measure the ribose and desoxyribose nucleic acid phosphorus (RNAP and DNAP) content of meristematic cells, and of the differentiated cells to which they give rise. The total phosphorus content of the same cell types and its distribution between acid soluble, lipid, and other forms, has been approximately estimated by Dr. Michael Ebert. The rate of uptake of inorganic phosphate was also measured.

The structure of *Vicia* roots (Fig. 1) lends itself to such measurements since transverse cuts at P, Q, R, S, T, U yield short segments of root populated predominantly by cells of a given type, as follows:

Segment A (PQ), 0–2 mm from the root cap. Apart from a small number (at most a few per cent) of differentiated epidermal cells, all cells in this segment are meristematic in appearance. The mitotic index among these cells under our culture conditions is known (3) to be around 15–20 per cent. It is customarily assumed that in this region both daughter cells from any given division divide again, but we have no positive evidence that this is so in all cases.

Segment B (QR), 2–4 mm. Cells in this segment are of two types, mixed in unknown proportions, *viz.* a) cells which will undergo one (or, rarely, more than one) division before commencing to form part of the differentiated root structure, b) cells which have already commenced to differentiate.

[1] Experimental work carried out while a member of the staff of the Medical Research Council's Radiotherapeutic Research Unit, Hammersmith Hospital.

[2] Nuffield Research Fellow of the British Empire Cancer Campaign.

Segment C (RS), 4–8 mm. Predominantly cortical cells and some vascular cells in process of active elongation, but containing cells of meristematic appearance, the two types being present in a roughly known proportion.

Segment D (TU), 12–20 mm. Predominantly cortical cells which have completed the process of elongation, and vascular cells, but mixed with a roughly known proportion of cells of meristematic appearance.

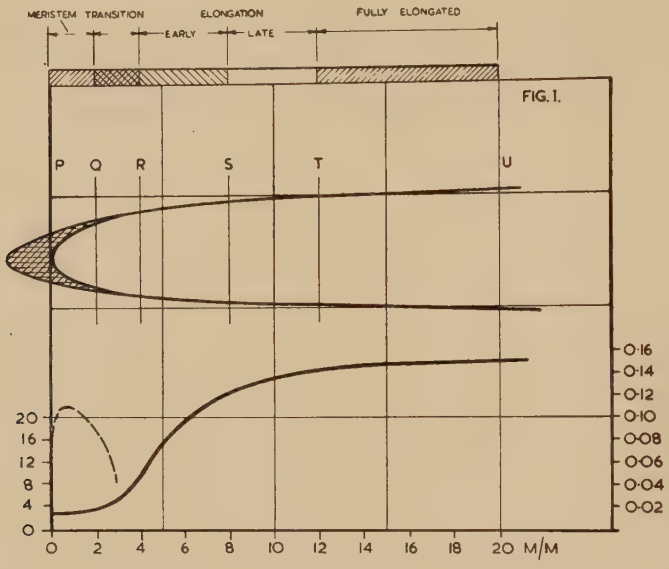

Fig. 1. Length of cortical cells and mitotic index in the segments of *Vica faba* root tips subjected to analysis. *Abscissae:* Distance from the tip after removal of root cap, in millimetres. *Ordinates:* Left-hand scale: Mitotic figures as percentage of total cell population. Right-hand scale: Mean length of cortical cells in millimetres.

EXPERIMENTAL METHODS

Seeds were germinated and seedlings grown in well aerated tap water at 19° C (Gray and Scholes, *loc. cit.*). Matched groups of roots—10 for cell counting and 40 for biochemical estimations—from a large number cultured simultaneously, were cut into segments one at a time under a low power binocular dissecting microscope by placing the roots in a V groove in a Perspex block hatched with fine lines at 2, 4 and 8 mm intervals. The root was cut by drawing a cataract knife through a gap in the Perspex block. The root cap, and the 8–12 mm segment (ST), were discarded. The remaining segments were transferred as they were obtained into ice-cold weighing bottles, stoppered to reduce evaporation. As far as possible, surface moisture was removed from each root by means of gauze before the root was cut.

Cell counting. The root segments were macerated for cell counting by the method of Brown and Rickless (1). 1 cc of 5 per cent chromic acid solution was added to the weighed material which consisted of segments of a given type from ten roots. It was left overnight and then diluted to 5 cc with chromic acid before maceration. Shaking was found to be insufficient to break up the segments, which were therefore drawn in and out of a hypodermic syringe through a serum needle until a suspension of cells was obtained. A drop of this suspension was run under the cover slip of a haemocytometer as in blood counting techniques.

Cell counts were normally made with a Burker haemocytometer of 0.1 mm depth. The depth is large compared with a meristematic cell, which is approximately 20 μ in diameter, but it is comparable with the length of a fully elongated cell. To ensure that no error was thereby introduced, check counts on the same cell suspension of 4–8 mm and 12–20 mm segments were made with a Fuchs haemocytometer of 0.2 mm depth, with the following results:

	Burker Haemocytometer	*Fuchs Haemocytometer*
Root segments, 4–8 mm	Cells/gm $1.30 \cdot 10^7$	Cells/gm $1.30 \cdot 10^7$
Root segments, 12–20 mm	Cells/gm $0.518 \cdot 10^7$	Cells/gm $0.513 \cdot 10^7$

Since the values obtained with the two haemocytometers were consistent, it is considered that no systematic error arose as between the counting of small and large cells.

The syringe method of maceration was continued in each case until the proportion of cells existing as clumps contributed only a little to the total count. A typical count from segment 2–4 mm, where cells are small, showed 84, 128, 9 and 20 cells in groups of 1, 2, 3 or 4 cells respectively. A similar count from the segment 12–20 mm, where cells are longer, showed 129 single cells, 6 cells in pairs and none in larger clumps. The number of cells in the horizontal divisions of the haemocytometer was always noted separately so that any non-random distribution would be obvious. More prolonged treatment with chromic acid did not diminish the cell count and there was no evidence of any loss of cells by the treatment.

Nucleic acid estimations. The weighed material, comprising segments of a given type from forty roots, was treated as follows:

The ordinary Schmidt-Thannhauser technique (12) was used. The tissues were ground in ice-cold 7 per cent trichloracetic acid, filtered, washed repeatedly with 7 per cent and afterwards with 1 per cent trichloracetic acid, and dried with ethyl alcohol and ether. The dried residue was extracted under a reflux condenser, first with boiling ethyl alcohol and ether (1:1 by volume), and next with boiling chloroform and methyl alcohol (1:1 by volume) to remove lipoids. The solvent was decanted and the residue allowed to dry, and was then incubated with N sodium hydroxide at 37° C overnight. From the solution obtained by this process the DNA was precipitated by a mixture of trichloroacetic acid and HCl, the precipitate was washed and the phosphorus was estimated by the method of Fiske and Subbarow (4). The phosphorus of the filtrate was also estimated and described as RNA phosphorus. It is known (3) that the small amount of phosphorus originally present as phosphoprotein is also found in this fraction and that it also contains small amounts of

unknown phosphate esters. We did not consider that the total amount of material with which we were dealing was sufficiently large to make separation of these substances a practical possibility. Since the DNA precipitates were very small, highly purified lactoglobulin was added to the solution in N soda to increase the bulk of precipitate. (It was also added to the "blank" determination.) If the separation of DNA and RNA had not been satisfactory, the addition of protein would have been expected to increase the efficiency of the separation. It did not, in fact, influence

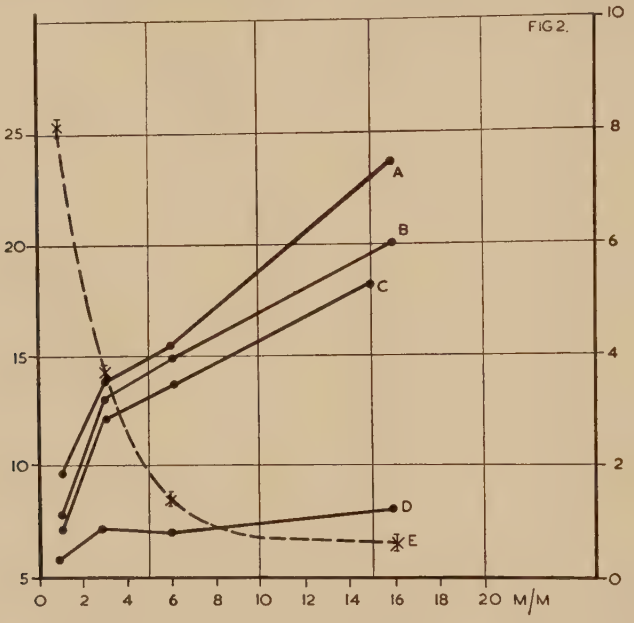

Fig. 2. Form and cellular content of the terminal 20 mm of root tip as a function of root length.
A) 50 roots, mean length 5.6 cm
B) 50 „ „ „ 10.3 cm
C) 280 „ „ „ 12.6 cm
D) 20 „ „ „ 22.6 cm
} Root form.
E) Cells per gram of wet tissue in each segment of roots approximately 10 cms in length.
Abscissae: Distance from the tip after removal of root cap, in millimetres. *Ordinates:* Left-hand scale: Wet weight per unit length of root in mg/cm. Right-hand scale: The number of cells per gram of wet tissue times 10^{-7}.

the estimated amounts of DNA and RNA, from which it was concluded that the ordinary procedure led to efficient separation. Protein was used in the experiment of 4–5/3/52.

It was found that, if glass tubes were used for incubation in N soda, a considerable depth of blue colour developed with the Fiske and Subbarow reagents in control estimations in which no tissue was present. Incubation was therefore carried out in wax-coated tubes. No sand or "Filter-aid" (Hyflo) was used during the extraction, as these substances also dissolved sufficiently in N soda to give a blue colour. These precautions were necessary on account of the small amount of material assayed.

TABLE I

Number of cells/gm in roots of *Vicia faba* $\times 10^{-7}$.

Seed	Date of Experiment	0–2 mm	2–4 mm	4–8 mm	12–20 mm
1949 stock	3/5/51	8.35±0.2	3.58±0.1	1.21±0.04	0.53±0.02
1949 ,, ...	2/8/51	7.5 ±0.4	3.6 ±0.3	1.43±0.03	0.69±0.06
1951 ,, ...	4–5/3/52	8.26±0.3	4.0 ±0.1	1.43±0.05	0.61±0.02
Pooled result		8.04±0.3	3.7 ±0.1	1.36±0.07	0.61±0.05

RESULTS

Estimates of the number of cells per gram of wet tissue. It is of interest to note the degree of consistency obtained in the cell counting procedures carried out on three separate occasions (Table I).

Standard errors of means of each individual experiment are inferred from replicate cell counts of aliquots from the same weighed sample of material by the usual procedure. Inter-experimental variations are significant ,since $\chi^2 = 53$ for $n = 11$, $P < 0.001$, but they are not large. It will be evident from Fig. 2 that since cell size varies so rapidly with distance from the apex, this rather high consistency in the estimates of the number of cells/gram can only be obtained by exercising great care in cutting the segments accurately to length. Since the form of the terminal 20 mm of root changes rather rapidly with root length (Fig. 2), it is also necessary in this type of estimation to match carefully, as to length, the roots used for cell counting and biochemical estimation.

The standard errors in the mean values of the number of cells/gram in the experiment of 4–5.3.52 were estimated at about 3–4 per cent.

Estimates of the nucleic acid content per gram. 240 roots were used to provide six replicate estimations of the DNAP and RNAP in the experiment of 4–5.3.52, which was considered to be satisfactory from the biochemical standpoint. The results are given in Table II.

In order to check the suitability of the Schmidt-Thannhauser procedure for the estimation of DNAP and RNAP in our material, a further batch of 50 roots were grown, and divided into two comparable groups. The terminal 20 mm was severed and the group of segments were weighed. The DNAP and RNAP of the one group were estimated by the Schmidt-Thannhauser procedure exactly as described above. In the other group the Ogur and Rosen perchloric method was used (10.) The results obtained were:

TABLE II

DNA and RNA content of root tips. Experiment 4–5/3/52.

	0–2 mm	2–4 mm	4–8 mm	12–20 mm
Estimates of the mgm DNAP per gm wet weight of tissue	0.312 0.323 0.307 0.324 0.250 0.250	0.186 0.171 0.147 0.154 0.152 0.155	0.0848 0.0717 0.0625 0.0512 0.0627 0.0590	0.0377 0.0305 0.0306 0.0220 0.0252 0.0260
Mean	0.294 ± 0.014 (4.8 %)	0.161 ± 0.006 (3.7 %)	0.0653 ± 0.0047(7.2 %)	0.0287 ± 0.0017(5.9 %)
Cells/gm $\times 10^{-7}$	8.26 ± 0.26 (3.1 %)	4.01 ± 0.13 (3.2 %)	1.43 ± 0.05 (3.5 %)	0.61 ± 0.02 (3.3 %)
DNAP/cell (10^{-12} gm)	3.6 ± 0.2 (5.7 %)	4.0 ± 0.2 (4.9 %)	4.5 ± 0.4 (8 %)	4.7 ± 0.3 (6.7 %)
Estimates of the mgm RNAP per gm wet weight of tissue	1.12 1.17 1.12 1.26 1.17 1.27	0.620 0.630 0.645 0.631 0.620 0.710	0.297 0.254 0.220 0.267 0.232 0.277	0.110 0.104 0.104 0.094 0.110 0.084
Mean	1.18 ± .03 (2.3 %)	0.643 ± .014 (2.2 %)	0.258 ± .011 (4.3 %)	0.101 ± .004 (4.0 %)
Cells/gm $\times 10^{-7}$	8.3 ± 0.3 (3.1 %)	4.0 ± 0.1 (3.2 %)	1.43 ± 0.05 (3.5 %)	0.61 ± 0.02 (3.3 %)
RNAP/cell (10^{-12} gm)	14.2 ± 0.5 (3.8 %)	16.1 ± 0.6 (3.9 %)	18.0 ± 1.0 (5.5 %)	16.5 ± 0.9 (5.2 %)

Standard error of the means, expressed as a percentage, are shown in brackets in each case.

	DNAP	*RNAP*
Schmidt-Thannhauser	0.070 mgm/gm	0.24_3 mgm/gm
Perchloric	0.078 mgm/gm	0.23_0 mgm/gm

We regard the agreements between the estimates of DNAP and RNAP obtained by the two methods as satisfactorily confirming the adequacy of the Schmidt-Thannhauser procedure for our purposes. If the amounts of DNAP and RNAP in the four segments of the root which are recorded in Table II and the amount in the 8–12 mm portion estimated by interpolation

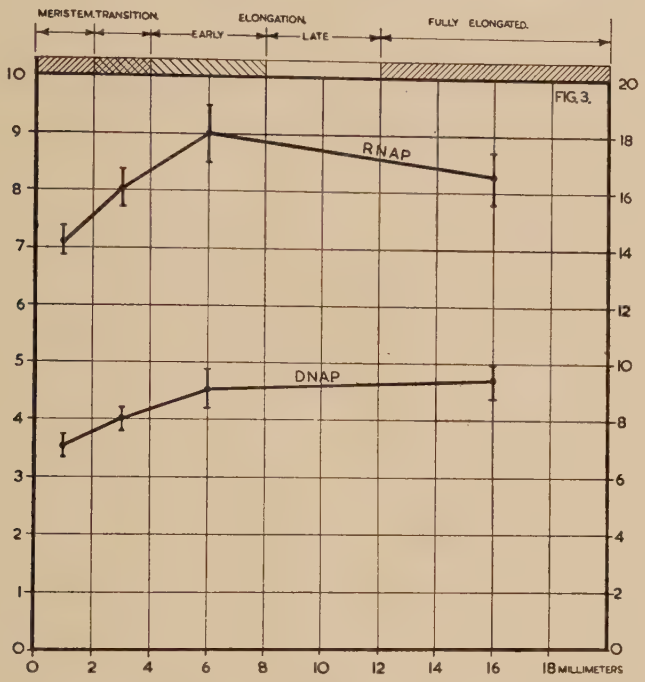

Fig. 3. The DNA and RNA content of *Vicia* root tips. *Abscissae:* Distance from the tip after removal of root cap, in millimetres. *Ordinates:* Left-hand scale: DNAP/cell in 10^{-12} gm. Right-hand scale: RNAP/cell in 10^{-12} gm.

are all added together, we obtain the following estimates of the amounts of DNAP/gram and RNAP/gram in the terminal 20 mm:

DNAP 0.071 mgm
RNAP 0.27 ,,

Thus, our check measurements not only show satisfactory agreement as between the Schmidt-Thannhauser and the perchloric methods, but also agree as closely as could be expected with measurements reported in Table II which were made two years ago on roots grown from a different crop of seed.

Estimates of total phosphorus, acid phosphorus and lipid phosphorus. (M. Ebert,

Medical Research Council, Experimental Radiopathology Research Unit.) The total phosphorus, acid soluble phosphorus and lipid phosphorus per cell in the four root segments was estimated to an accuracy of 20 per cent, which was all that was required to give an approximate indication of the amount of phosphorus in these various forms in each of the four segments, for comparison with the DNAP and RNAP estimates with which we were primarily concerned. Root segments were prepared exactly as for DNAP and RNAP analysis. The roots were from the same stock and of approximately the same length as those used in the experiment of 4–5/3/52, but were not grown at the same time and therefore not matched with these as to growth rate.

After the roots had been cut, segments were kept near 0° C until analysed. For total phosphorus estimations, root tips were heated in 5 : 1 H_2SO_4, $HClO_4$ mixtures, the cold and clear solutions partly neutralised with NaOH, and the phosphorus content measured. The acid soluble phosphorus was extracted from the root tips by pulverising them in an agate mortar below freezing point, adding cold 5 per cent trichloracetic acid to the powder and filtering through a sintered glass filter. The phosphorus content of the filtrate was estimated. The lipid phosphorus was extracted by heating the intact root tips for thirty minutes in a 2 : 1 alcohol ether mixture, evaporating the solvent, digesting the residue with 5 : 1 H_2SO_4, $HClO_4$ and partly neutralising the acid. The phosphorus estimations were carried out by converting the inorganic phosphate to $(NH_4)_3PO_4 \cdot 12MoO_3$, reducing this with 1 : 2 : 4 aminonaphtholsulphonic acid at 25° C and measuring the blue colour formed in a Unicam spectrophotometer at 675 mμ and .1 mm slit width 15–20 minutes later. Blanks without roots were run simultaneously. The results of these measurements are presented as a histogram in Fig. 4.

Tracer studies. Measurements of the uptake of P^{22} (5) have shown that when roots are cultured in tap water containing 16 mg of NaH_2PO_4 per litre, all but 1.5 per cent of the phosphorus content of all four segments is derived from the cotyledons. With decreasing concentration of phosphorus in the culture medium, the amount of phosphorus contributed from the culture medium falls proportionately below 1.5 per cent and is negligible under our conditions of culture.

DISCUSSION OF RESULTS

It is apparent that both the mean DNAP per cell and the mean RNAP per cell increase somewhat (and in about the same proportion) as the meriste-

matic cell undergoes transformation into a fully differentiated cell. Applying the χ^2 test to the data given in Table II:

Mean DNAP/cell = 4.00; $\chi^2 = 11.6$; $n = 3$; $P = 0.01$.
Mean RNAP/cell = 15.6; $\chi^2 = 14.2$; $n = 3$; $0.01 > P > 0.001$.

The increase is therefore statistically significant.

The results depicted in Fig. 3 are contrary to those which we anticipated on the basis of the assumptions 1) that the DNAP content of the cells investigated consists entirely of an invariant amount associated with each fully formed chromosome, and 2) that all differentiated cells are diploid. If both these assumptions were true, the mean DNA content of the meristematic cells would be expected to be greater than that of the differentiated cells because, during the period of synthesis, the DNA associated with the chromosomes of the meristematic cells is increasing above the diploid value $(DNA)_{2n}$, and after the completion of synthesis remains at 2 $(DNA)_{2n}$ till the completion of telophase. In *Vicia* roots grown under our conditions, mitosis lasts about four hours, and Howard and Pelc (8, 9) have presented very strong reasons for believing that the incorporation of phosphorus into the fully polymerised DNA molecule is complete eight hours before the onset of mitosis. Thus for twelve hours out of a complete cycle of thirty hours, the DNA content per cell is 2 $(DNA)_{2n}$, and the mean content (associated with the chromosomes) must exceed $\frac{1}{30}[18(DNA)_{2n} + 12 \times 2\,(DNA)_{2n}] = 1.29\,(DNA)_{2n}$. If we allow for a linear increase throughout the synthetic period, then the estimated mean value is 1.5 $(DNA)_{2n}$.

Thus, if all cells in segment A (0–2 mm) were truly meristematic, and all cells in segment D (12–20 mm) were fully differentiated, we should expect the ratio of DNAP/cell in these two segments to be as 1.5:1. In fact, cells of meristematic type are to be found in segments C and D. In the intact root they occur in association with the vascular bundles, and by proliferation at a slightly later time will give rise to lateral roots. When the cell populations from each segment were being counted in the haemocytometer, cells were designated as meristematic or differentiated on the basis of their size, and scored separately. The chromic acid treatment used to prepare the tissue for reduction to a cell suspension was too drastic to permit a classification of cell types on the basis of cytological structure. It was found that in segment D, 'meristematic' cells constituted about 23.7 ± 1.3 per cent of the whole. Because cell size was the only criterion of cell type, it is certain that this figure overestimates—possibly to a considerable degree—the proportion of cells which are preparing for further division. On the basis of this estimate,

however, we may adjust the figure for the ratio of expected DNA/cell in segments A and D to 1.5 $(DNA)_{2n}$ $[0.24 \times 1.5\ (DNA)_{2n} + 0.76\ (DNA)_{2n}]$ or 1.34 : 1. Since, in arriving at this ratio, we have almost certainly overestimated both the proportion of truly meristematic cells in the more proximal segments and their rate of turnover, the expected ratio must exceed 1.34. The presence of non-dividing cells in segment A, about which we have no certain knowledge at all, would, of course, reduce the ratio towards unity. It would be very astonishing if as high a proportion as half the segment A cells were non-dividing. The expected ratio can therefore hardly be less than 1.17. Since the observed DNAP content of the segment A cells is $3.57 \pm 0.2 \cdot 10^{-12}$ gm, a mean value for the cells of segment D greater than $3.1 \pm 0.1_7 \cdot 10^{-12}$ gm is certainly incompatible with our two original assumptions. Values greater even than $2.6_7 \pm 0.1 \cdot 10^{-12}$ would be doubtfully compatible. The observed value is $4.7_0 \pm 0.3_2 \cdot 10^{-12}$ gm. The differences are 1.6 ± 0.36 and 2.0 ± 0.36 respectively, and are therefore highly significant on a purely statistical basis ($P = 10^{-6}$ and 10^{-7} respectively).

With a view to maintaining a roughly constant standard of accuracy in the estimations, the amount of tissue used for analysis (*i.e.*, the relative length of the segments) was chosen so as to yield roughly equal amounts of DNAP. Attention has, however, been drawn to the fact that the DNAP is always a small fraction of the total phosphorus, and is, moreover, a diminishing fraction in proceeding from segment A to segment D (Fig. 4). The ratio of DNA to other cell constituents also varies from segment to segment. Such circumstances could have introduced systematic differences in the efficiency with which DNA was separated from other phosphorylated compounds.

While recognising this as a possible source of error, we have no reason to invoke it, and must rather infer that one or both of our original assumptions are invalid. The unexpectedly high relative content of DNA in the proximal segments could be due to the presence of a substantial proportion of polyploid cortical cells, or to the presence of DNA additional to the amount computed on the assumption of a fixed quota associated with each fully synthesised chromosome of given type.

The conclusion has been reached, on cytological grounds (10), that it is the rule rather than the exception for the chromosome complement of a proportion of nuclei of differentiated root tissue to be double, or some higher multiple of the diploid number. Thus, in the elongation zone of roots, $4n$ cells have been found in nineteen species, and $8n$ cells in nine species out of 21 examined and listed by D'Amato. *Vicia faba* is among those in which $4n$ cells have been found, but these seedlings have not been so fully inves-

tigated as many other plants. Estimates of the proportion of polyploid cells in the cortex range from about 10–20 per cent in *Nothoscordum fragrans* to 95–100 per cent in the 'pull-roots' of *Bellevalia romana*.

By the photometry of Feulgen stained nuclei, Patau (11) finds many cells in the elongating zone of the root tip of *Rhoeo* which have twice the diploid amount of DNA, and a few with four times this amount. In the elongation

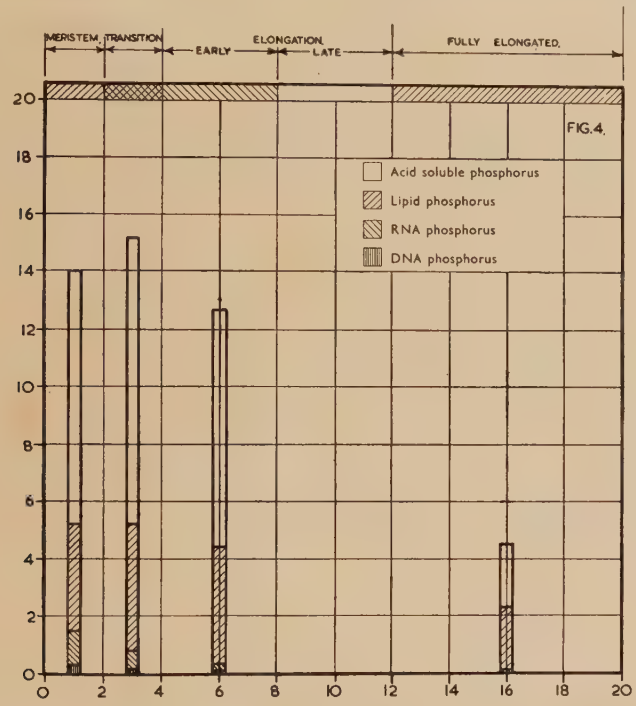

Fig. 4. Composition of 4 segments of *Vicia faba* root tips with respect to different forms of phosphorus. *Abscissae:* Distance from the tip after removal of root cap, in millimetres. *Ordinates:* Phosphorus content in mg P/gm wet weight.

zone of *Zea mays* roots, Swift (13) finds nuclei having from twice to sixteen times the diploid amount of DNA. If cells with higher multiples of the diploid amount of DNA occurred in *Vicia* differentiated cells with the frequency observed by Swift in *Zea*, the expected DNAP content of segment D would be $10.3 \cdot 10^{-12}$ gm/cell, compared with our observed value of $4.7_0 \pm 0.3 \cdot 10^{-12}$ gm/cell. It is thus evident that on the basis both of cytological and of photometric evidence, the composition of the nuclei in the elongating zone of some species is such that a mean DNAP/cell relative to meristematic

tissue as high as, or higher than, we have observed in *Vicia faba*, is to be expected.

Our data represent a condition in which the *mean* content of the fully differentiated cells is about twice the diploid amount. It is interesting to note that this value is already attained by cells only 4–8 mm from the tip which are in the early stage of elongation.

The fact that the DNA content of the differentiated cells is greater than the mean DNA content of the meristematic cells, and probably on an average about equal to the tetraploid amount, implies that in this material the mechanism which controls the cessation of cell proliferation and that which controls the cessation of DNA synthesis are not uniquely coupled. A considerable proportion of cells near the proximal end of the meristem evidently synthesise DNA but fail to undergo a further division.

We hope that more precise information for *Vicia faba* will soon be available. Photometric studies on Feulgen stained *Vicia faba* meristematic cells have just been reported by Grundemann and Marquardt (7), and comparative measurements on cells from the four segments which we have investigated are being undertaken by Deeley, Davies and Chayen.

Discussion of the problem which formed the starting-point of our studies, namely, the relation between the actual DNA content of meristematic and differentiating cells and the rate of incorporation of P^{32} into the DNA of these cells, as revealed by autoradiograph studies, is therefore deferred till the results of the photometric measurements are available.

SUMMARY

By combined cell counting and biochemical assay, the mean DNA and RNA content of the cells in the root tip of *Vicia faba* seedlings has been measured. The values for segments 0–2, 2–4, 4–8 and 12–20 mm behind the root cap were found to be, for DNAP, $3.5_7 \pm 0.2_0$, 4.00 ± 0.20, $4.5_5 \pm 0.3_7$ and $4.7_0 \pm 0.3_2$, 10^{-12} grams, and for RNAP, 14.2 ± 0.54, 16.1 ± 0.63, 18.0 ± 0.99, and 16.5 ± 0.86, 10^{-12} grams. The approximate amounts of phosphorus per cell in other forms were also estimated. The nucleic acid determinations show that the mean DNA content per cell steadily increases with distance from the root apex, being 30 per cent greater in the proximal segment, which is composed mainly of fully differentiated cells, than in the terminal segment, which is entirely meristematic. Thus, if the average meristematic cell at the commencement of interphase is assumed to have a

diploid content of DNA, then the mean DNA content of the differentiated cells is approximately tetraploid.

We wish to thank Dr. M. Ebert for his co-operation, and in particular for carrying out the estimation of acid soluble, lipid, and total phosphorus, and Miss Diana Lorimer for technical assistance.

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Experimental Cell Research, **8**, *114–117* (*1955*)

STUDIES ON MITOCHONDRIA IN EARLY CLEAVAGE STAGES OF THE SEA URCHIN EGG

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As shown in an earlier paper (6) the visible differentiation in the sea urchin larva is shortly preceded by a dramatic rise in the number of mitochondria, cf. Fig. 2 in the present paper. This result is in agreement with the increase in enzyme activity (5) and acetate metabolism (7) that occurs in these stages. During the cleavage stages, on the other hand, the mitochondrial number is low and appeared to be constant according to a rough estimation. In the present paper, the mitochondrial number in these early stages has been the subject of a quantitative study.

The method used in the present work is essentially that described in our earlier paper (6). The fertilized eggs, cleavage stages, and early blastulae were subjected to vital staining with Nile blue sulphate and gently pressed below the cover glass by slowly removing some sea water with the aid of filter paper. During this process the individual blastomeres were closely packed against each other and against the fertilization membrane (which might eventually burst) and the blastocoel was reduced to a narrow slit. Thus, the objects for counting had the appearance of circular cakes. The microscope was focused on a certain level in the cytoplasm and the distinctly stained intracellular bodies were counted with the aid of a network inserted in the ocular. The sample sector evidently corresponded to a cytoplasmic plate the thickness of which was determined by the depth of focus of the microscope. The side of the sector was about a third of the diameter of the "cake". The polarity of the eggs in the early developmental stages is sometimes difficult or impossible to determine, and the germs were thus oriented by chance. In those stages where the orientation could be discerned, no local differences in the frequency of stainable particles could be observed. In a 32-cell stage the sample sector in the animal region had the relative density value of 11.6 whereas the value from the vegetal sector was 12.8, i.e. not significantly different from the animal value. This is in agreement with our previous observations which show that a mitochondrial gradient does not

appear until the advanced blastula stage. The sample sector thus appeared to be representative for the whole germ, and single test sectors from the different stages of development should consequently give information about the changes in the relative number of stainable particles. The particles varied in size, and one could expect that it would be difficult to decide upon the minimum sized particle which should be counted. Nevertheless counts of

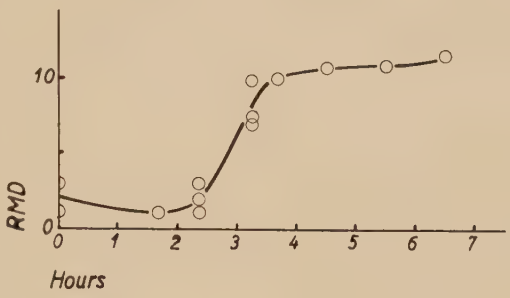

Fig. 1. Changes in the relative number of Nile blue sulphate stainable granules during early cleavage and blastula stages of the sea urchin. Individual counts of two separate experiments plotted together. RMD = relative density of the particulate population. Sample sectors chosen by chance with regard to the polarity of the egg. 32-cell stage was reached at 3 hours 15 minutes.

the same sector by one of the authors essentially agreed with the counts of the other observer. On the other hand the values tended to rise if the counting was made very slowly, probably since particles of smaller dimensions and lower stainability were then counted. In spite of this and other sources of error the results from different series of determinations were consistent.

The mitochondrial development appears to take place by a gradual aggregation of elements (1, 2, 8, cf. 4). It is probable then that the particles counted represent only a fraction of the developing particulate units within the sample sector, notably the largest one. It is also possible that the staining procedure has accelerated the formation of particles from material which is still in the soluble state.

The results of the determinations are summarized in the accompanying curves. The scatter is much less than one might expect with this crude method, and bearing in mind that each point corresponds to one separate larva. There is no conflict between the different curves if they are compared on the basis of cleavage stage. Fig. 1, in which the individual counts of two experiments are plotted, shows that the number of stainable mitochondria remains low during the first two to three hours of development (at about 21° C). It shows a subsequent rise of about the same duration and finally reaches a rather constant level. Determinations on separate batches of eggs starting on the 32 cell stage (Fig. 2 a) and on the young blastula stage respectively (Fig. 2 b) show that this constant level is followed by an abrupt rise in the

advanced blastula, i.e. the rise described in an earlier paper (6). This latter rise is greater in the animal region than in the vegetal (cf. Fig. 2 b).

Data are lacking as yet to permit an accurate reconstruction of the course of the curve from the fertilized egg to the gastrula stage. It is, however, evident that such a reconstructed curve would have resemblance to the respiratory curve of Lindahl (9) and to the changes in intensity of the iso-

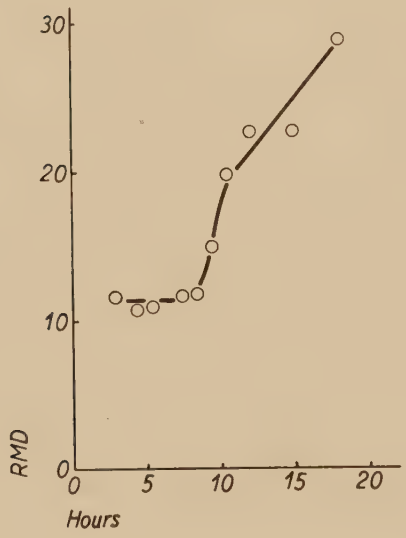

Fig. 2 a.

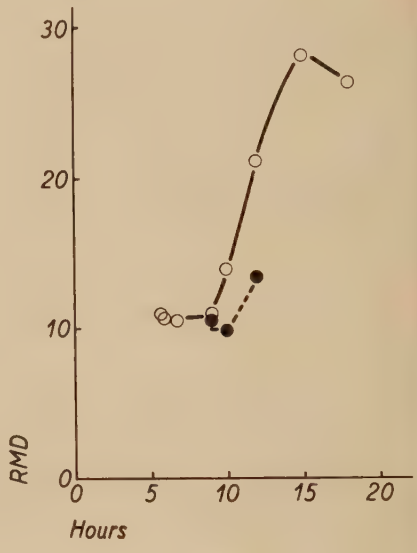

Fig. 2 b.

Fig. 2 a and b. Changes in the relative density of the mitochondrial population (RMD) in the blastula and young gastrula of the sea urchin. Sample sector chosen by chance with regard to the polarity up to the 6 hour stage, thereafter placed in the animal region (open circles), or in the vegetal region (filled circles). 32-cell stage was reached at 2 hours 30 minutes and hatching between 6 and 7 hours.

tope incorporation from C^{14} acetate into free hypoxanthine given by Hultin (7). The rise in the number of stained particles is earlier, however, than the rise in the metabolic curves, but all these curves probably describe phenomena which are intimately related to each other. It is to be noted in this connection that enzymes studied so far in the sea urchin development (5) do not increase their activity markedly until the advanced blastula or during gastrulation.

The mitochondrial development evidently cannot proceed to any great extent during cleavage stages. The nucleus probably has to choose between the reorganization of itself and the control of syntheses in the cytoplasm as

suggested by Zeuthen (10). Mitochondria may even, reversibly, lose the major part of their substance during the mitotic phase according to Frédéric and Chèvremont (3). The rise in the number of particles during the cleavage stages may thus reflect the increasing number of persisting nuclei in a similar way as the respiratory activity seems to, as suggested to the authors personally by Zeuthen (cf. 10). The question then arises why the curve is leveling off and remains at a plateau for some hours before it shows the final strong rise. The idea has been expressed (4) that such diapauses may be due to the depletion of the cytoplasm in some compounds which were accumulated in the unfertilized egg.

SUMMARY

The mitochondrial development during the cleavage stages of the sea urchin egg has been studied. An S-shaped increase in the number of stainable particles was demonstrated. This increase has been compared with the rise in the number of persisting nuclei and various metabolic changes studied by other authors.

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OBSERVATIONS ON DIFFERENTIATION WITH INHIBITED CLEAVAGE IN THE SEA URCHIN EGG

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Sea urchin eggs removed from the ovaria are frequently in a state of cytoplasmic underripeness. This often holds true for the eggs of *Psammechinus miliaris*, the shallow form, collected at the Zoological Station of Kristineberg on the Swedish west coast. The underripeness is generally not very pronounced. However, it may be difficult to get a normal high elevation of the fertilization membrane, unless the eggs are repeatedly washed or treated with glycine (7). If the sperm is added immediately after the eggs have been removed from the ovaria, the fertilization membrane often sticks to the egg surface. When this occurs, plasma cleavage during subsequent development is often suppressed although the nuclear divisions are more or less undisturbed. Such eggs develop into syncytia possessing well developed cilia enabling the larvae to swim around after hatching. Similar larvae have been obtained by Runnström (personal communication) in eggs from which the jelly coat had been removed by means of acid treatment, and which consequently adhered to the bottom of the glass container with their sticky surfaces. In both these cases the suppression of the plasma cleavage may be due to mechanical factors, notably the adherence of the egg to a rigid surface. Partial suppression of plasma cleavage in the sea urchin egg (*Paracentrotus lividus*) has also been obtained by means of treatment with CO and with reduced oxygen pressure (4). A differentiation without cleavage has also been described for various annelid worms (for ref., see 4), and ascidians (for ref., see 1).

Differentiation with inhibited plasma cleavage was obtained in nearly 100 per cent of the eggs in a batch from *Psammechinus miliaris* which was fertilized immediately upon removal from the ovaria. At a stage when the larvae would have normally been gastrulae, the syncytia presented the appearance of an uncleaved egg, although of a smaller diameter. The question arose whether the inhibited cleavage and morphological differentiation in such syncytia was paralleled by an inhibition of the mitochondrial develop-

ment. Cytological studies were performed on vital stained material in this stage (Nile blue sulphate according to the prescriptions made by Gustafson and Lenicque (3)). Quantitative comparisons between the syncytia and normal larvae could not be performed on account of their different shape. It was, however, evident that the number of mitochondria and their general appearance were about the same.

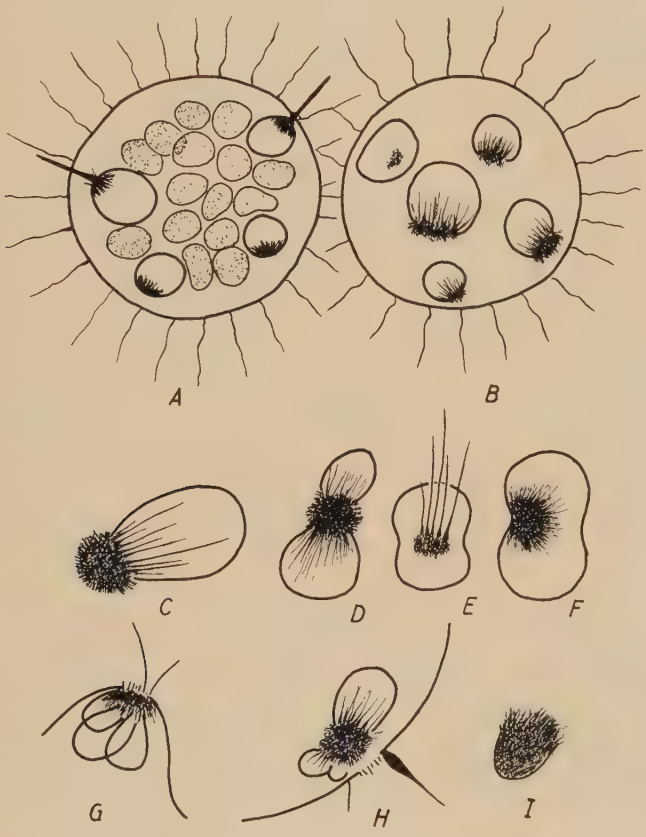

Fig. 1. Differentiation with inhibited cleavage in *Psammechinus miliaris*. A and B, larvae 20 hours old; C–H details of vacuoles and fibrous inclusions, from the central parts of the larvae (C–F), and from the peripheral parts with fibrous rods protruding from the larval surface (G–H); I, detail of a cluster of fibrous elements.

The cytoplasm of the syncytia always contained a series of large vacuoles of various size (Fig. 1 A–H). A system of smaller vacuole-like plasma-filled structures which occupied the spaces between the big vacuoles were observed in some of the syncytia, but only in the central part of the larvae (Fig. 1 A). It has not been determined whether these structures are the result of an incomplete suppression of plasma cleavage, or if they are giant nuclei formed, e.g., by fusion of several nuclei, cf. (4). The syncytia also contained big

bundles or clusters of fibrous elements, always adjacent to the vacuoles, which themselves appeared to be formed by water squeezed out from the fibres (Fig. 1 A–I). Certain fibrous elements or bundles of elements attained sufficient length to run straight through a vacuole (Fig. 1 C) and even continue on its other side (Fig. 1 E). Occasionally, the thick fibres or rods protruded from the outer surface of the syncytium (Fig. 1 A, G, H).

These observations indicate that mitochondrial development takes place even when plasma cleavage is suppressed. This is in agreement with a series of observations which show that a respiratory rise occurs in the syncytial development of *Chaetopterus*, *Urechis*, and *Ciona*, although the rise is more or less retarded (1, 2, 6).

The morphogenetic development of the sea urchin egg can be resolved to some extent into a series of localized cell-stretchings which stepwise transform the thick-walled blastula into a series of thin-walled vesicles. A cell-stretching in one area is always preceded by the development of mitochondria in that area. It was suggested (3) that the mitochondria produce fibrous structural proteins which form a framework in the cells and thus make it possible for them to attain and retain a flattened shape. The formation of these fibrous elements may thus be an observable link between the morphological and the biochemical level (cf. 5). It has not yet been possible to demonstrate such fibrous elements in normal larvae (except for the animal tuft of stereocilia). This may be because the fibrous elements are utilized and incorporated, e.g., in the cytoskeleton of the cell walls, at the same rate as they are produced. In the syncytia of *Psammechinus* there are no cell boundaries and thus there is no matrix for the fibrous elements to be deposited on. Consequently the fibrous elements aggregate and accumulate in thick bundles or clusters from which water is squeezed out. The present material is unusually favourable for observing the formation of intracellular fibrous elements. On the other hand no rigid cytoplasmic compounds have been demonstrated in the syncytia of the ascidian *Ciona* (1).

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THE EFFECT OF EXCESS VITAMIN A ON MITOSIS IN CHICK HEART FIBROBLASTS *IN VITRO*

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In recent work, excess vitamin A was found to produce interesting effects on organ cultures. Fell and Mellanby (1) demonstrated that the limb bone rudiments of embryonic chick and foetal mouse disintegrated if cultivated in A hypervitaminotic medium while the surrounding soft tissue grew profusely. Studer and Frey (3) and Sabella *et al.* (2) described a thickening of the epidermis due to increased proliferation of the *stratum granulosum* in rats treated with excess vitamin A, both systemically and locally.

These results raised the question of how cell division is influenced by A hypervitaminosis. To investigate this point it was decided to grow a series of chick fibroblast cultures with excess vitamin A and study its effect on mitosis by comparing cell counts in treated and untreated cultures.

MATERIAL AND METHODS

The cultures were obtained from the hearts of 10–11 day old chick embryos and were transplanted at least three times before treatment. They were then halved, one half to serve as experimental culture, the other to be kept as control. Before and during the experiment the hanging drop technique was used, i.e. the explants were grown on glass cover slips in a medium consisting of one drop of fowl plasma and one of chick embryo extract. Experimental cultures received plasma containing 4000 i.u./100 ml, so that the final medium contained half that amount while the control plasma had a concentration of 200 300 i.u./100 ml. Most of the vitamin in the artificially reinforced plasma was in a fat-soluble form, in contrast to the natural vitamin which is water-soluble (for details see (1)).

Experimental and control cultures were maintained *in vitro* for up to seven days from the beginning of treatment and transferred to fresh medium every 2 days.

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[1] Sir Halley Stewart Fellow.

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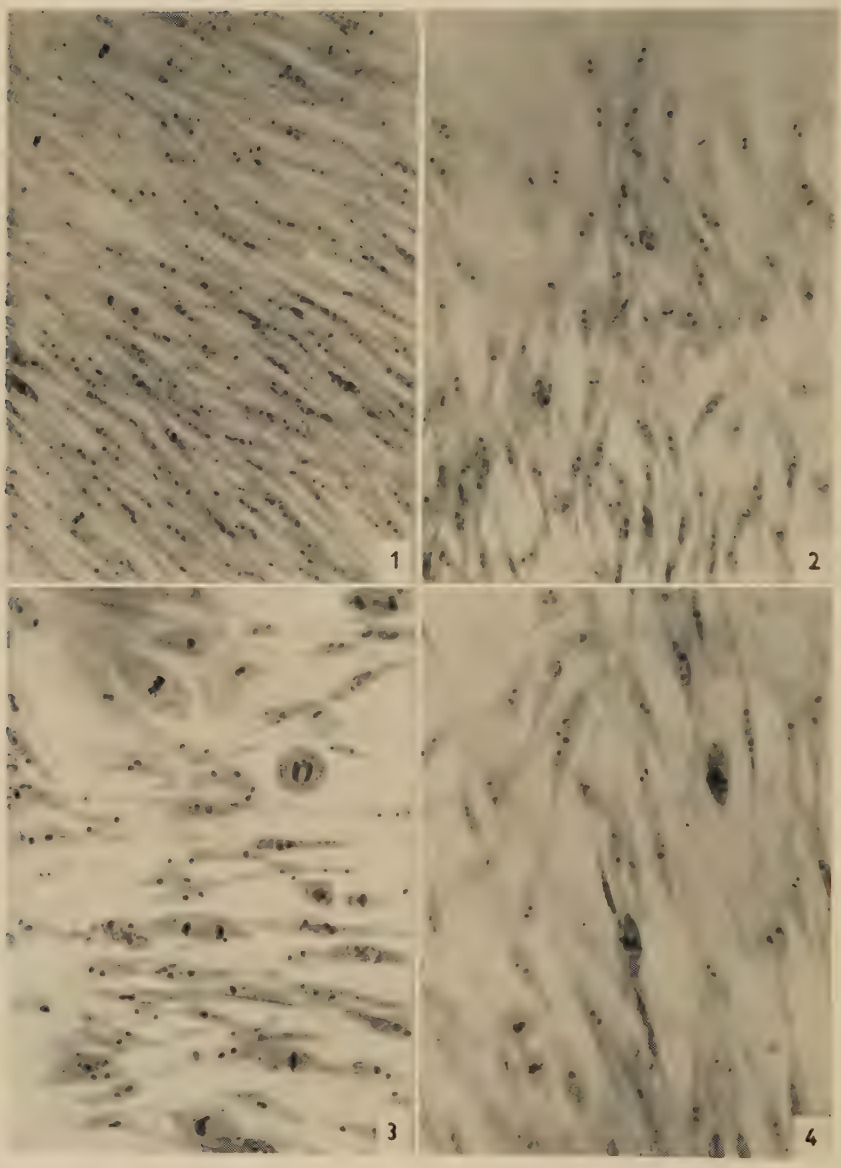

Fig. 1. Edge of culture grown for 7 days in A medium showing dense outgrowth. Haematoxylin. × 350.
Fig. 2. Comparable field in control culture. Haematoxylin. × 350.
Fig. 3. Mitosis in culture treated for 7 days with excess vitamin A. Note abundance of cell division. Haematoxylin. × 480.
Fig. 4. Mitosis in control culture. Haematoxylin. × 480.

During transfer the identity of the original pairs was preserved. They were fixed in Susa solution 2, 4, 5, 6 and 7 days after the beginning of the experiment, and stained with haematoxylin as whole mounts. Five to six pairs were used for each point of observation. Cell counts were made by selecting comparable fields in treated and untreated cultures and counting all cells, resting and mitotic, present. Approximately 500 cells were enumerated in each culture, and mitosis was expressed as the percentage of resting cells. In cultures fixed after 2, 5 and 7 days the total number of cell divisions was also counted in treated and untreated cultures and the results expressed as the percentage of the experimental over the control counts.

RESULTS

Under normal conditions the central explant of fibroblast cultures becomes surrounded with a network of radially directed cells after 24 hours' incubation. During the next day, this zone of growth enlarges and mitotic activity usually begins after 6 hours' incubation, reaching a peak at 20 to 30 hours. From this time, the number of cell divisions falls, and on the second day of growth mitosis may represent only half the value seen during the peak period.

Up to and including the fourth day of cultivation no difference could be observed between experimental and control cultures as regards mode of growth, architecture and density of the cell colonies, or in cytological structure. The final size reached by the outgrowth before transfer was similar in both sets of cultures. On the fifth day of the experiment, however, the cell density was slightly greater in treated cultures. This difference in cell density became more pronounced by the seventh day when the fibroblasts in A+ medium appeared as bundles of closely packed, slender and elongated cells (Fig. 1), while in control cultures they were arranged in a more loosely knit meshwork (Fig. 2), and were more flattened, broader, and, especially at the periphery of the culture, often assumed a triangular shape. The areas occupied by the zone of growth was larger in A+ medium.

Table I gives the results of the mitotic counts, expressed as a percentage of resting cells and of the total number of cell divisions. Up to and including the sixth day of the experiment, the mitotic rate is equal in A+ and in normal medium, but there is a rise in the total number of cell divisions on the fifth day. On the seventh day, the mitotic rate is increased by 50 per cent in treated cultures while the total mitotic count is doubled (Figs. 3 and 4). The difference in the mitotic index and the total mitotic count reflects the increase in cell density in A+ medium and indicates that the promotion of growth is due to cell division as well as to cell migration.

CONCLUSIONS

In vitro, vitamin A has different effects depending on the type of tissue treated and on whether it is unorganised or differentiating. Thus in the experiments described above, a dose of vitamin A which causes rapid disintegration of bone and cartilage *in vitro* (1) had no inhibitory action on the

TABLE I

Effect of excess vitamin A on mitosis.

Days after beginning of experiment	Normal medium		A+ medium		Percentage A+/Co of total mitosis
	Mitotic index	Average number of cell divisions per culture	Mitotic index	Average number of cell divisions per culture	
2[1]	8.7	143	7.6	130	85± 7.9
4[1]	5.6±0.5		6.9±0.58		
5	10.0	116	10.4	157	140±17.2
6[1]	4.5		4.9		
7	6.4±0.51	77	9.6±0.6	148	201±16.3

unorganised growth of embryonic heart fibroblasts, and the treated cultures showed more mitosis and greater outgrowth than the controls in normal medium. Whether the excess vitamin produced a genuine stimulation of growth in the treated cultures or whether it merely prevented the diminution of mitosis in the controls (see Table I) due to inadequate supplies of the vitamin in the normal medium, is uncertain. It is clear, however, that the vitamin is closely concerned with the mitotic activity of the tissue.

The alteration of cellular shape in treated cultures is probably not a direct effect of the vitamin but secondary to the increased cell density observed.

SUMMARY

Chick heart fibroblasts were grown in a medium containing 2000 i.u. of vitamin A for periods of up to 7 days. Batches of treated and untreated cultures were fixed after 2, 4, 5, 6 and 7 days' growth and their mitotic index and total mitotic count determined. During the first 6 days, the size and mitotic rate of cultures grown in A+ medium were the same as in the controls. Cytological changes were absent. By the seventh day, the mitotic rate

[1] Subcultivation of remaining cultures.

had risen by 50 per cent and the total number of cell divisions was doubled in the treated cultures while the cell density and size of outgrowth were increased. The results could be interpreted in two ways: as a genuine promotion of growth by the vitamin, or as inhibition of the control cultures due to insufficient supplies of the vitamin in normal medium.

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QUANTITATIVE DETERMINATION OF DESOXYRIBONUCLEIC ACID IN ECHINODERM GERM CELLS[1, 2]

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In a previous publication, desoxyribonucleic acid (DNA) was quantitatively determined in a suspension of *Arbacia punctulata* eggs using the method of isotope dilution with C^{14}-labelled thymine (23). By this procedure the small amount of DNA in the preparation could be determined with a high degree of accuracy and it could be shown that much, if not all, of it could be accounted for outside of the ovum itself in the polar bodies and in the syncitial cells. The frequency of syncitial cells could not be determined with the same precision as the DNA analysis. It had also been shown that the nucleus of the mature ovum of *Arbacia* and *Asterias* contained no detectible Feulgen-positive material (22, 23), altho such material was present in the oogonia and young oocytes. Nuclei of the late oocytes (germinal vesicle stage) were Feulgen-negative, but this latter observation could be explained either as an absence of DNA or as dilution of the DNA in the relatively huge volume of this nucleus to the point where it was not detectible with the microscope. Consequently it was considered advisable to attempt to isolate the nuclei of oocytes in this stage in order that a direct chemical analysis of their DNA content could be made. The results to be described have shown that there is ample DNA in these nuclei of *Asterias* and hence that the negative Feulgen reaction in this instance was due to dilution of the DNA in the nucleus. However this interpretation of the absence of Feulgen-positive material could not be applied to the very much smaller nucleus of the mature ovum.

Preliminary observations on the sea urchin *Tripneustes ventricosus* indicated that eggs of this species were free of the syncitial cells found with *Arbacia* eggs, but it was found that there were a considerable number of

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DNA-containing bodies having their origin in a slimy substance secreted from the surface of the animal, which became attached to the extruded eggs and were not removed by repeated washing. However it was possible to determine rather accurately the amount of DNA attributable to these bodies. As in *Arbacia* and *Asterias*, the nucleus of the mature *Tripneustes* egg is Feulgen-negative altho the nucleus itself is small and its chromatin strands are not diffuse or attenuated. Chemical analyses by the isotope dilution method using C^{14}-thymine and the cytological observations lead to the conclusion that the nucleus of the mature *Tripneustes* egg contains very little or no DNA.

MATERIAL

Asterias forbesii sperm. For Batch I the gonads were removed from 6 males and the shed sperm collected from these in a small amount of sea water. The suspension was diluted to 1 liter with filtered sea water, filtered through muslin and then bolting cloth. The filtrate was centrifuged at 500 g for 5 minutes in an International refrigerated centrifuge to remove debris, and the sperm was then sedimented from the low speed supernatant at 1000 g for 15 minutes. The sedimented sperm was suspended in filtered sea water, passed through cheese cloth, then diluted to 500 ml. One ml aliquots were removed in duplicate from the homogeneous sperm suspension to obtain sperm counts. These were diluted with sea water and 10 ml of formalin to a final volume of 100 ml. The remainder of the sperm suspension was centrifuged at 1000 g for 50 minutes to sediment the sperm quantitatively. Salt was removed from the sperm by washing with 53 per cent ethanol in distilled water and the sperm recovered after centrifugation for 60 minutes at 1000 g. Lipids were removed by extracting at room temperature with acetone-absolute ethanol 2:1 twice over a 6-day period, with absolute ethanol-ether 3:1, 3 times over a 10-day period, with methanol-chloroform 2:1 twice during 3 days, then with petroleum ether twice for 24 hours. The sample was dried in air and then to constant weight at 78° C during a 4-hour period. The weight of the dry sample was 8.8567 grams. Sperm counts were done in a hemocytometer giving a total of 3.025×10^{12} sperm cells for the 500 ml of sperm suspension.

Since the procedure used for the collection of nuclei from oocytes, to be described in the next section, involved exposing them to sucrose solutions, another batch of *Asterias* sperm (Batch II) was collected and treated as follows to test for possible losses of DNA to the sucrose solution. The sperm was washed and foreign material removed as described above for Batch I, then divided into 2 equal portions. To the first, serving as a control, an equal amount of 95 per cent ethanol was added to coagulate the sperm, and then left in the cold. To the other aliquot of sperm in 75 ml of sea water an equal amount of 0.5 M sucrose was added and centrifuged immediately in the cold at 1000 g for 1 hour. The supernatant (sup. 1) was set aside and the sediment reextracted with the same volume of 0.25 M sucrose for 59 hours in the cold. The suspension was then centrifuged for 2 hours in the cold at 1000 g giving supernatant 2. The sediment (se-2) was coagulated with 6 volumes of 95 per cent ethanol.

Supernatants 1 and 2 were each coagulated with 5 volumes of alcohol and the sediments (3 and 4) so obtained were lipid-extracted and dried as described for sperm, Batch I. The control, which was left in the cold for 65 hours, was sedimented at 1000 g for 75 minutes, coagulated with alcohol, lipid-extracted and dried as described previously. The yields of the lipid-free residues were as follows: control, 3.38 g; se-2, 3.18 g; se-3, 0.177 g; se-4, 0.085 g.

Nuclei from Asterias forbesii oocytes (germinal vesicle stage)

Five preparations were collected. Preparations 1 and 2, which were later pooled for analysis, were isolated in the following manner: 4 females were rinsed in tap water and the ovaries removed to 4 bowls 10 inches in diameter containing about 200 ml of a solution of 0.25 M sucrose and 0.0018 M $CaCl_2$. In this medium, which has been used by Hogeboom *et al.* (14) for the isolation of cell fractions from mammalian tissue, the oocytes are shed but they do not go through the maturation divisions. When observed microscopically, some of the intact germinal vesicle nuclei can be seen being extruded from the oocytes. After 1 hour in sucrose at room temperature, during which most of the eggs were shed, the ovaries were stirred in a Waring blendor using a rheostat to give a very low speed. This mild agitating served both to increase the yield of oocytes from the ovaries, and to break the oocytes with minimum damage to the nuclei. The volume of sucrose was reduced by siphoning off the clear supernatant and the suspension was filtered through muslin. The filtrate was placed in an ice bath and layered over a solution of 0.34 M sucrose and 0.0018 M $CaCl_2$ in 50 ml cups at 0° C. These were centrifuged for 3 minutes at 500 g in an International refrigerated centrifuge. The top layer contained cytoplasm and egg membranes and was discarded. The bottom portion, consisting mostly of germinal vesicle nuclei and nucleoli with some egg membranes, was taken up in 0.34 M sucrose[1], filtered through muslin, then bolting silk (160 threads to the inch) to remove egg membranes, and the filtrate was recentrifuged at 500 g for 3 minutes. The sediment was made up to a known volume in 0.34 M sucrose, and hemocytometer counts done on a suitable aliquot. The remaining nuclei were sedimented at 900 g for 5 minutes in the cold. The sediment was coagulated with cold 95 per cent ethanol, and after 2 more cold washings in this medium the sedimented material was lipid-extracted at room temperature and then dried as described for the *Asterias* sperm preparation. The types of nuclei found and the dry weights of the lipid—extracted powders obtained are given in Table I.

Preparations 3, 4, and 5 were not subjected to centrifugation in order to minimize the yield of broken nuclei as indicated by the high nucleoli count for preparations 1 and 2. The eggs were shed into 0.20 M sucrose in these preparations since it was found to give better yields than 0.25 M sucrose. Sedimentation of the nuclei by gravity in the cold was used to separate them from the cytoplasm which remained concentrated in the supernatant, the cytoplasmic clumps settling much slower than the nuclei. In a typical preparation, the eggs suspended in 0.20 M sucrose were ruptured by stirring mechanically in a beaker using a T-shaped glass stirring rod or blended at very low speed as described for the previous preparations, and the nuclear fraction

[1] When $CaCl_2$ was omitted, no differences were detected in the nuclei isolated.

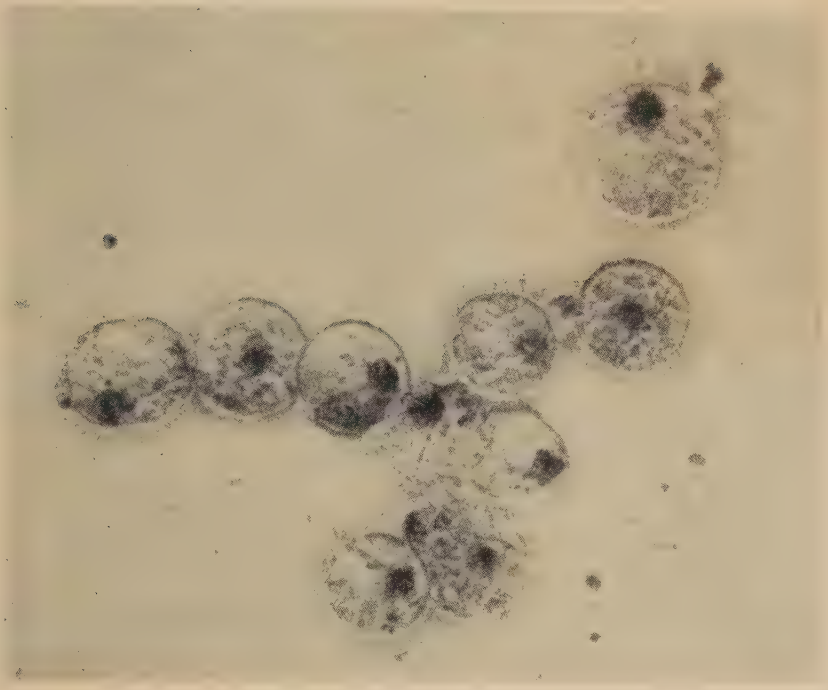

Fig. 1. Nuclei in the germinal vesicle stage isolated from *Asterias forbesii* eggs. Note deformation of three of the nuclei and folds in the membranes of the deformed nuclei. Photograph taken with phase contrast.

TABLE I

Nuclear preparations from *Asterias* oocytes (germinal vesicle stage).

Preparation	Distribution per 100 nuclei counted			Number of nuclei[1] $\times 10^7$	Dry weight in mg
	Large nuclei	Small nuclei[2]	Nucleoli		
1	36	18	46	3.72	235
2	68	10	22	2.75	375
3	80	5	15	0.89	142
4	92	3	5	3.23	939
5	81	4	15	2.58	293

[1] The totals include nucleoli as well as whole nuclei. This procedure was used because the sediment contained in addition to whole nuclei and nucleoli some partially collapsed nuclei which lacked nucleoli. The free nucleoli were therefore taken as a measure of the number of such distorted nuclei.

[2] Nuclei with diameters approximately $^1/_2$ or less than that of the maximum size were put into this category.

was sedimented by gravity for 12 hours in the cold. The sediment was resuspended in 0.25 M sucrose, left for 24 hours in the cold and the sediment again collected, suspended in 0.25 M sucrose and filtered through bolting silk to remove egg membranes. The sediment was again resuspended in 0.25 M sucrose and the filtration repeated, the bottom fraction collected and made up to a known volume for nuclear counts. The appearance of the isolated nuclei is illustrated in Fig. 1. The yields after lipid extraction and drying are given in Table I. Preparations 3, 4, and 5 were then pooled for analysis. The fraction of the egg material which was removed in the isolation of the nuclei could be estimated from the dry weight of the eggs from which they were obtained. The latter values were calculated from the known size and density of the eggs, making the assumption that the water content was 90 per cent. The calculations showed that about 95 per cent of the egg material had been removed in the preparation of the nuclei.

Separation of the RNA and DNA was then carried out on the two batches of pooled nuclei as previously described (23). The lipid-free residues were digested in N KOH for 18 hours at 30° C with continuous stirring, using 1 ml KOH solution for each 100 mg of lipid-free dry powder. To the digests a mixture of 3 N trichloracetic acid (TCA) and 12 N HCl were added in the proportions 0.2 ml of the mixture for each ml of KOH solution used. After 1 hour, the mixtures were centrifuged to separate the precipitates containing the DNA and the supernatant fluids containing the RNA nucleotides. The sediments were washed twice with 0.125 N TCA, the washings being combined with the RNA fractions. The sediments were then extracted twice with 10 per cent TCA for 15 minutes at 90° C. The soluble and insoluble portions were separated by centrifugation. After decomposition of the TCA in the extracts at 100° and removal of water in a stream of N_2, the dried residues which weighed 44.8 and 121 mg respectively were collected for digestion and analysis.

Tripneustes ventricosus sperm

Fresh sperm was obtained from males of this species by adding 0.5 ml of 0.53 M KCl to the body cavity after decanting most of the perivisceral fluid. The concentrated sperm suspension was diluted with filtered sea water and filtered through 8 layers of cheese cloth. After a low-speed centrifugation for 2 minutes to remove tissue particles and clumped sperm, the sperm was sedimented by centrifugation for 5 minutes at high speed in a table model International centrifuge. The sedimented sperm was diluted with sea water to 100 ml, filtered through bolting cloth and resedimented. The cycle was repeated, and the final sperm suspension made up to 100 ml with sea water. For counting, one ml of the sperm suspension was diluted to 90 ml with sea water, one ml of formalin added dropwise with stirring and the volume made to 100 ml. Counts were made in a hemocytometer. The mean deviation of counts from 3 separate dilutions was 1.3 per cent. The remaining 99 ml of the sperm suspension, corresponding to 4.43×10^{11} sperm cells, was sedimented by centrifugation and then suspended in 50 per cent ethanol at 0° C for 48 hours. The coagulated sperm was then collected by centrifugation, washed 2 times with 50 per cent ethanol and 4 times with a mixture of acetone-absolute ethanol 2:1, the final washing giving a colorless supernatant. The pellet obtained was stored in a desiccator over $CaCl_2$. Before analysis, the dried pellet was ground in a glass mortar and pestle, and the

lipids more completely removed by extraction with 4 changes of a mixture of methanol and chloroform 2:1 for 30 hours and then with petroleum ether 2 times for 17 hours. The petroleum ether was removed and the fat-free sperm powder dried to constant weight in an oven at 70°. The weight of this preparation was 1.5110 grams.

Tripneustes ventricosus eggs

Eggs were obtained from 25 females by causing them to shed into sea water in the same manner as described above for the collection of sperm. The eggs were diluted with sea water and distributed among 10 bowls of 10 inch diameter. After the eggs settled by gravity, the supernatant water was siphoned off and this procedure was repeated. The eggs were then collected into 4 bowls of the same size and the washing procedure repeated three times with filtered sea water. The eggs were then left for 1 hour in filtered sea water, passed through 4 layers of cheese cloth into 1 bowl and samples taken from about the middle of the suspension for examination. Microscopic examination showed 2 small oocytes in the 894 mature eggs counted. An aliquot of the egg suspension diluted further with sea water, placed in a finger bowl and examined microscopically 4 hours later showed no late oocytes and no eggs in cleavage. The eggs were distributed into 4 large bowls and washed with filtered sea water 3 more times. They were then fixed and extracted as follows, all procedures being carried out at 5–10° C. A thick suspension of the eggs in sea water was diluted with an equal quantity of a mixture of 5 per cent citric acid in distilled water and sea water, 1:1. The supernatant fluid was removed from the eggs which settled out by gravity, and an amount of 5 per cent citric acid in distilled water added equal to the volume of the residual egg suspension. This last step was repeated 2 times, and the 285 ml of egg suspension left overnight in the last citric acid solution. Most of the citric acid solution was then removed and the volume of the egg suspension brought to 1800 ml with 70 per cent ethanol. After 2 hours the supernatant was again removed and 95 per cent ethanol was added to the same volume. This alcohol was changed 9 times during a 63-hour period. After the 8th washing in 95 per cent ethanol, an aliquot of the eggs was removed for staining, using the Feulgen procedure with the appropriate controls as previously described (22). After the ethanol extractions the eggs were suspended in acetone-absolute ethanol 2:1 for 12 hours and then in acetone. The eggs were then stored in acetone. The eggs were divided into 2 batches which were counted separately. One batch representing about one half of the total was analyzed in this work and contained $1.435 \pm 0.050 \times 10^8$ eggs where 0.050 is the standard error.

Fraction of the Tripneustes eggs

Fraction 1. To obtain the acid soluble fraction the suspension was transferred quantitatively to a tared 250 ml centrifuge bottle. The acetone supernatant was removed by centrifugation and the eggs suspended in 150 ml of ice cold 5 per cent TCA, stirred for 10 minutes then centrifuged for 10 minutes in the cold. This procedure was repeated twice. The combined supernatants were a pale amber solution which was faintly opalescent.

Fractions 2 and 3. To remove traces of TCA from the eggs and to complete the lipid extraction, the eggs were treated with ice cold 70 per cent ethanol, cold 95 per

cent ethanol twice, cold acetone twice and finally with the lipid solvents as described in the section on *Asterias* sperm. The weight of the residue, after drying to constant weight, was 4.05 grams. This was subjected to treatment with 1 N KOH for the separation of RNA and DNA and the subsequent isolation of the DNA fraction was carried out with 10 per cent TCA at 90° in the same manner as described previously. The fraction containing the RNA nucleotides, together with the 0.125 N TCA washings of the sediment, was designated as fraction 2. The 10 per cent TCA extract was then heated to decompose the TCA and the volume of the solution reduced in a stream of nitrogen at 100° C. The residue, fraction 3, taken to constant weight at 100° C was 0.4687 grams.

DIGESTION AND ANALYSIS

Asterias sperm

Three hydrolysates were made from the sperm of Bath I, one on a 10.0 mg sample and the other two on 100.0 mg samples, using 70 per cent $HClO_4$ for digestion (24). After centrifugation to remove the carbonized material, 24.05 μl aliquots of each were deposited on paper chromatograms. Following chromatography with butanol-HCl (24), the concentrations of thymine were determined in the distilled water eluates of the paper.[1]

This sperm powder was also used to test the efficiency of extraction of DNA with hot TCA. Two 100.0 mg portions and one 300.0 mg portion were digested with KOH in the usual way and the insoluble residue resulting after acidification were extracted with 10 per cent TCA at 90° for 15 minutes, 1 ml of TCA used for each 100 mg of powder. The insoluble residues were reextracted with half the amount of TCA for another 15 minute period and these extracts combined with the first ones of each sample. The TCA was decomposed and water removed in the usual way. The residues were digested with $HClO_4$ and suitable aliquots deposited on paper. The amounts of thymine found after chromatography in butanol-HCl, elution with distilled water and quantitative spectrophotometry, expressed as the percentage of thymine in the original sperm powder, was 1.46, 1.50 and 1.45, with 1.47 as an average. Since direct analyses of the sperm powder as described above gave 1.73 per cent thymine, 85 per cent of the thymine is recovered under these conditions. Therefore, when this procedure was applied to the other preparations the values obtained were corrected for this 15 per cent loss.

Of the fractions collected in Batch II sperm, se-4 was analyzed for its thymine content after hydrolysis of 10.0 mg of this material with 70 per cent $HClO_4$. After chromatography of 24.05 μl of the digest, no thymine could be seen on the chromatograph in ultraviolet light, or detected by spectrophotometry in an eluate from the hydrolysate lane of the region opposite a standard thymine spot on a parallel lane on the same chromatograph. Since 1 γ of thymine would be detected both on the chromatograph and in the spectrophotometer reading after elution, we can calculate the upper limit of the possible loss of thymine, or DNA, from the sperm to the sucrose solution when this material remained in contact with the sucrose over an

[1] It should be stated that a series of preliminary tests showed that after chromatography, thymine was quantitatively extracted from the filter paper with distilled water.

extended period as described previously. It was thus calculated that less than 0.22 per cent of the total thymine content of the sperm could have been extracted by the sucrose.

Asterias oozyte nuclei

PREPARATIONS 1 + 2. The DNA residue was digested with 70 per cent $HClO_4$, an aliquot of the solution streaked on a paper chromatogram, and the bases separated in the butanol-HCl system. The thymine band was readily seen, as well as the other bands which absorb ultraviolet light which remain near the starting line in this solvent.[1] The thymine was purified by elution with distilled water, the eluate concentrated in a stream of N_2 and rechromatographed in the same solvent mixture. The distilled water eluate of the thymine, in which the concentration of thymine was determined, gave a curve in the spectrophotometer that agreed with the standard curve for thymine.

PREPARATIONS 3—4—5. After digestion of the DNA fraction with 70 per cent $HClO_4$, the hydrolysate was streaked on 3 chromatograms, and the thymine regions eluted with distilled water after chromatography in butanol-HCl. The eluates were pooled and concentrated as described previously, and the thymine purified by successive runs in butanol-NH_3 and butanol-HCl. The concentration of thymine in the final eluate was calculated from the spectrophotometric curve, as above, the curve obtained agreeing with the standard curve for thymine in the region 230–300 mμ.

The RNA fraction obtained from these combined preparations was tested for the presence of thymine. The slightly opalescent supernatant and washings of the precipitate which resulted after the HCl–TCA mixture was added to the clear KOH digest of the powder were pooled and left in the cold for 2 weeks. A small amount of precipitate which formed at the end of that time was collected and analyzed separately from the supernatant. The supernatant was heated at 100° C to decompose the TCA. The solution was then taken to dryness by vacuum distillation at 37°. HCl was removed by the addition of distilled water followed by evaporation *in vacuo*. The residue was dissolved in distilled water and the potassium removed by the formation of the insoluble perchlorate in the cold. The precipitate was washed, the washings combined with the solution, water removed in a nitrogen stream and the residue digested with 70 per cent $HClO_4$. The digest was streaked on to three chromatograms and chromatographed in the butanol-NH_3 solvent. Altho ultraviolet absorption bands of the other bases were found, no absorption was observed in the thymine regions of the chromatograms. The latter were pooled, however, extracted with distilled water and the extract concentrated, placed on a chromatogram in a small drop and rechromatographed in butanol-HCl. No thymine could be detected in this of the uracil region which was similarly chromatographed. Since 1 γ of thymine would be visible under these conditions, less than this amount or 5 per cent of the final thymine value found for this pooled preparation is the upper limit for possible losses of DNA to the RNA fraction under these conditions.

The precipitate described previously which had been formed in the cold was extracted with 10 per cent TCA at 90° C and the extract decomposed, concentrated

[1] No attempt was made to identify and estimate the other purine and pyrimidine bases of DNA which were present in these bands.

and digested with 70 per cent $HClO_4$. No thymine was detected in this fraction after chromatography in butanol-NH_3. The thymine region was pooled for rechromatographing with the similar region of the other chromatograms of the RNA fraction in which no thymine could be detected in the final chromatogram as described above.

Tripneustes sperm

Two aliquots of the sperm powder were digested for 1 hour in 12 N $HClO_4$ in 12 ml open centrifuge tubes and thymine isolated by chromatography with butanol-NH_3. The yields were 37.3 and 38.1 μg thymine per mg powder. Two other larger aliquots of approximately 300 mg were digested for 5 hours with 2 N $HClO_4$ in similar tubes which in this case were fitted with cold fingers to prevent concentration by evaporation of water. Measurement of the solution volume before and after digestion showed that evaporation had in fact been prevented. The yields were 35.7 and 35.6 μg of thymine per mg powder, or about 95 per cent of the values obtained with 12 N $HClO_4$.

Tripneustes eggs

Fraction 1. The TCA was decomposed by heating, the volume reduced under N_2 and the material taken to dryness. The dried residue, 0.1155 g, was digested with 70 per cent $HClO_4$ as previously described. After removal of the insoluble residue from the digest, 1 N KOH was added slowly to the digest in an ice bath to neutralize the perchloric acid. After removal of the $KClO_4$ the volume of the solution was reduced under N_2 to 0.3 ml which was then subjected to chromatography with butanol-HCl. The region in the digest lane opposite the standard thymine spot was eluted and again subjected to chromatography. No thymine could be found.

Fraction 2 (RNA). Since the amount of thymine expected in the eggs was too small to be detected by the usual methods of direct analysis it was considered that the possibility of the loss of some of the DNA to this fraction could be more readily tested with the oocyte nuclei obtained from *Asterias*, as described in a previous section. From the results obtained, the method of separation of RNA and DNA appears to be adequate. In a previous study on *Arbacia* (23) the RNA fraction was tested for the presence of thymine and none could be detected.

Fraction 3 (DNA). From digestion studies with *Tripneustes* sperm described previously it was found that comparable yields of thymine were obtained by digestion for 1 hour in 12 N $HClO_4$ and for 5 hours in 2 N $HClO_4$. To minimize the volume of $KClO_4$ precipitate, the latter procedure was used on the DNA residue from the eggs. After the digestion was completed, C^{14}-thymine prepared as previously described (23) was added, the mixture thoroughly stirred and allowed to stand overnight. The undigested sediment was removed by centrifugation and the volume of the supernatant fluid reduced in N_2 to 0.75 ml. This was deposited as a streak on each of three paper strips and chromatographed using the butanol-HCl system. The regions opposite the thymine spots were eluted with distilled water, the eluate concentrated in N_2 and again subjected to chromatography in butanol-HCl. This process was then repeated using the butanol-NH_3 system, followed by 3 more runs in butanol-HCl. The absorption spectrum of the final eluate agreed with that of the thymine standard in the region 250–290 mμ with the maximum deviation being

TABLE II

Thymine content per single *Asterias* germ cell.

Sample	Mg thymine per 100 mg dry weight, lipid extracted powder	Number of nuclei or sperm per 100 mg powder	μg thymine per nucleus or sperm	Thymine in oocyte relative to sperm
Sperm, Batch I . . .	1.73	3.402×10^{10}	5.09×10^{-8}	1
Asterias Oocyte Nuclei				
Preparations 1+2	3.40×10^{-3}	1.06×10^{7}	3.21×10^{-7}	6.27
Preparations 3+4+5	1.52×10^{-3}	4.88×10^{6}	3.12×10^{-7}	6.07

less than 2 per cent. C^{14} was measured on the same eluate in a gas flow Geiger counter. A single Levy-Lang pipette with a delivery volume of 24.05 μl was used in all C^{14} measurements. This volume of solution was deposited on clean metal planchets and dried under an infra red lamp. Three aliquots of the final solution and three of the original C^{14}-thymine were counted. In each case a total of about 50,000 counts were taken, the minimum counting rate being approximately 65 times the background count.

RESULTS

Asterias

The results obtained on the thymine content per single *Asterias* germ cell are given in Table II. The data obtained for the two analyses done on *Asterias* oocyte nuclei preparations agreed to within 3 per cent, although the preparations differed in the per cent of nucleoli found. The result indicates that the method of counting each nucleolus as representing a nucleus in the preparation is valid. The amount of thymine found in the oocyte nuclei was about 6 times the amount of thymine per sperm of this species.

Tripneustes

From the amount of thymine found and from the counts of the number of sperm cells in the preparation it was calculated that there was $12.14 \pm 0.15 \times \times 10^{-8}$ μg thymine per *Tripneustes* sperm. The amount of thymine in the *Tripneustes* egg preparation was calculated from the dilution of the specific activity of the C^{14}-thymine added. The specific activity of the C^{14}-thymine added, with a maximum deviation of 1.16 per cent for 3 determinations, was 4,020 counts per minute per μg. The amount of thymine added was 30.66 μg. The specific activity of the thymine recovered from the digest was 1,389 counts per minute per μg with a maximum deviation of 1.57 per cent

for 3 separate determinations. With these data the amount of thymine originally present in the digest (y) was obtained as follows:

$$\frac{4020}{1389} = \frac{30.66 + y}{y} \qquad y = 57.95\ \mu g$$

Since the tests with *Asterias* sperm showed that with the procedures applied 85 ± 2.0 per cent of the thymine present was recovered in the hot TCA extract, the above value is subject to a correction of 15 per cent. Allowing for the 15 per cent loss the amount of thymine in the preparation containing 1.435×10^8 eggs was 68.18 μg.

As mentioned previously, no syncitial or "nurse" cells were found when a thorough examination of an aliquot of the living egg suspension was made with the phase contrast microscope or after Feulgen staining of fixed preparations. However, the latter showed that a considerable number of Feulgen-positive bodies 20–25 μ in diameter were present either attached to the surface of the eggs or occasionally in small masses independent of the eggs. These bodies were homogeneous in appearance and did not show the usual chromatin structure of nuclei. For 992 eggs examined 508 of these bodies were found, that is, a proportion of these bodies to eggs of $0.512 \pm .016$. Since *Tripneustes* when irritated secretes a colorless water-soluble slimy substance from its surface which might be included in the egg preparation in spite of repeated washing, it was examined as a possible source of the Feulgen-positive bodies. Slime was collected separately from male and female animals and precipitated in alcohol. The precipitate was then subjected to the usual Feulgen staining procedure. An aliquot washed free of alcohol was digested for two hours at 30° C with a solution of crystalline pancreatic desoxyribonuclease.[1] The undigested slime from both males and females had embedded within it numerous Feulgen-positive bodies of the same size and general appearance as those previously found associated with the eggs. The slime treated with the desoxyribonuclease and stained in the same way contained similar bodies, but these were colorless. As a control, rat liver nuclei with and without desoxyribonuclease were digested with HCl and stained at the same time as the two slime preparations. The enzyme-treated nuclei were colorless while those not so treated were intense magenta in color. Most of the stained Feulgen-positive bodies of the slime and the eggs were optically homogeneous but occasional ones contained strands suggesting chromatin. Some observations made with mature *Arbacia eggs* may be instructive in this connection. After a suspension of such eggs in sea

[1] From Worthington Chem. Co.

water, layered over 0.88 M sucrose, was centrifuged at 78,000 g, nuclei contained in the small "white halves" showed typical chromatin structure altho they were not stained when treated by the Feulgen method. The same preparations also contained many free nuclei, apparently released from ruptured cells, which were also Feulgen-negative but had no detectible chromatin structure.[1] It therefore appears most likely that the Feulgen-positive bodies are nuclei extruded from somatic cells along with more or less cytoplasm, which have had their typical chromatin structure altered by contact with sea water. As somatic nuclei it would be expected that they would contain twice the amount of DNA found in the sperm cell. There seems to be general agreement among those making such measurements (2, 31) that in a variety of species of animals the sperm has the haploid amount of DNA.

Using the above information it becomes possible to allocate the thymine found as follows. The number of Feulgen-positive bodies in the preparation analyzed is $1.435 \times 10^8 \times 0.512$, and the amount of DNA-thymine in these bodies may be calculated as: $1.435 \times 10^8 \times 0.512 \times 2 \times 12.14 \times 10^{-8} = 17.846$ μg. Since the amount found in the preparation was 68.18 μg the remainder, 50.33 μg, may be assigned to the eggs and their polar bodies, i.e. 35.07×10^{-8} μg thymine for each egg and its polar bodies. This quantity is 2.89 times the amount found per sperm. If the egg cell as well as its polar bodies contained the haploid amount of DNA-thymine, it would be expected that the egg and its polar bodies would have 4 times the amount in sperm. Stated in another way, the amount found is just sufficient to account for the DNA in the polar bodies leaving none that could be assigned to the egg itself. An alternative distribution of the 35.07×10^{-8} μg of thymine equally to all members of the meiotic tetrad may be made. By application of this procedure one obtains as a result 8.77×10^{-8} μg of thymine in each cell, or about 70 per cent of the amount in sperm. However, this method of assignment is not deemed to be justified, for reasons which will be given in the discussion.

As mentioned previously, no thymine could be found in a digest of the material obtained on extracting the *Tripneustes* eggs with cold 5 per cent TCA, altho under these conditions as little as 1 μg could be detected. If thymine had been present in an amount equal to that of sperm, 17 μg should have been found. The results therefore suggest that the thymine, or any low molecular weight compound containing it, which may have been present was removed during the extraction of the eggs with cold 5 per cent citric

[1] It may be mentioned that yolk granules had been removed by centrifugation and that yolk granules give a "plasmal" reaction to the Feulgen stain, which the nuclei do not. Nuclear preparations from *Asterias* oocytes (germinal vesicle stage).

acid prior to the extraction with TCA and lipid solvents. It should be mentioned that nuclei of eggs fixed either in 5 per cent citric acid or in a mixture of ethanol and acetic acid were Feulgen-negative, while sperm in the same fixatives were stained with equal intensity. Studies on mammalian tissue have shown that cold 5 per cent citric acid does not extract DNA (19). Therefore the assumption that DNA was lost in the citric acid solution used in the preliminary treatment of the eggs would not be justified.

DISCUSSION

In a previous investigation of *Arbacia* eggs it was concluded that most of the DNA found could be accounted for outside of the nucleus of the egg cell. The nucleus of the egg was Feulgen-negative and arguments were presented for considering that the failure to observe the stain could not be attributed to dilution of DNA either by dispersion in an exceptionally large nuclear volume or over the surface of the nucleus. Nuclei of oogonia and oocytes prior to the germinal vesicle stage were Feulgen-positive (23). Nuclei of young oocytes of *Tripneustes* are also Feulgen-positive and, like *Arbacia*, the nuclei of the mature eggs are Feulgen-negative. As in *Arbacia*, the chromatin strands are readily seen in the nucleus of the mature egg and are not expanded to unusual size. The same situation with respect to staining was found to exist in *Asterias* eggs. Brachet has reported (3) that following centrifugation of eggs in the germinal vesicle stage, Feulgen-positive chromatin can be found at one side of the nucleus altho the nuclei in the non-centrifuged eggs appear to be Feulgen-negative. We have found that following centrifugation with a force as high as 78,000 g for 20 minutes there is no such accumulation of the Feulgen-positive material in the nuclei of the mature *Arbacia* eggs. The results of our experiments on the effects of sucrose on *Asterias* sperm show that it is unreasonable to postulate that there could have been any appreciable extraction of DNA by sucrose from the nuclei of the *Arbacia* eggs during centrifugation. In nuclei as small as these one would expect that, even if they contained somewhat less DNA than the sperm, following compaction by centrifugation such DNA would be detectible by the Feulgen stain. It follows from these observations that if there is any DNA at all in the nuclei of the mature eggs of *Arbacia*, *Asterias*, and *Tripneustes*, the amount must be considerably smaller than that in the sperm.

From the results of the analyses with C^{14}-thymine it is possible to give a precise account of the amount of acid-insoluble thymine, i.e. the thymine presumed to be in DNA, that may possibly be present in the egg cell. We

consider the use of thymine as an index for DNA justified by the observation that *Tripneustes* sperm, like that of the other echinoderm species investigated, contains thymine in the usual amounts, and by the fact that the comparisons here made are given in terms of the thymine found in the sperm DNA. A thymineless DNA in the *Tripneustes* egg may not be reasonably invoked as an explanation for the absence of thymine, not only because no such DNA has as yet been found, but the negative Feulgen reaction would remain unaccounted for. In a series of investigations with a variety of materials, including echinoderm eggs and sperm, it has been found that digestion with 70 per cent perchloric acid as we carry it out does not give any detectible destruction of thymine (21, 24, 32).

The major uncertainty in our experiments lies in the manner of assignment of the thymine found to the members of the meiotic tetrad in the female, i.e. the egg and its polar bodies. Altho on genetic grounds one may assume equal distribution of genetic materials to each member of the tetrad, postulating a similar distribution of DNA is tantamount to the assumption that DNA is the primary genetic material or a necessary and ever-present constituent of it. As an adjunct of the genetic material it is of course possible for the DNA to be either present or absent. On the former assumption, with equal distribution of the DNA found, each member of the tetrad would contain only 70 per cent of the haploid complement as determined in the sperm. This deficiency of 30 per cent in the amount per haploid nucleus is about 32 times the standard error of the determination and hence very unlikely to occur on the basis of probability. Furthermore this equal distribution could not be justified by the cytological findings, for if the egg nucleus did actually contain 70 per cent of the haploid amount of DNA it should be microscopically visualized with the Feulgen reaction either with or without centrifugation. Another mode of distribution of the DNA-thymine found is, therefore, required. We have found that in the three echinoderm species investigated the nuclei of the polar bodies are Feulgen positive as long as the polar bodies can be seen. If by analogy with the data obtained by other investigators on the DNA content of the members of the male meiotic tetrad in a variety of species of animals and of plants (3) the haploid quantity of DNA-thymine is assigned to each of the three polar body nuclei, none remains for assignment to the egg. Any other method of assignment would be contrary to the observations made on this as well as other species. Thus, with respect to DNA, the meiotic process is essentially similar in the male and female in producing an equal distribution to the members of the meiotic tetrad, but the egg pronucleus divests itself of its DNA. Of course, the con-

clusion that there is no DNA in the egg nucleus may be drawn only within the limits of error of the experimental determinations.

The standard error in the determination of the total amount of DNA-thymine is quite small, i. e. 0.46 per cent. The standard error of the number of eggs counted is 3.12 per cent and that for the proportion of contaminating Feulgen-positive bodies to eggs is 3.10 per cent. Thus the minimum amount of thymine that might be allotted to these bodies is given by:—

$$(1.435 - 0.045) \times 10^8 \times 12.14 \times 10^{-8} \times 2 \times [0.512 - (0.512 \times 0.031)] = 16.74\ \mu g.$$

The maximum total amount of thymine is given by:—

$$[57.95 + (57.95 \times 4.6 \times 10^{-3})] \times 1/0.85 = 68.49\ \mu g.$$

Then the maximum amount in eggs plus polar bodies is: $68.49 - 16.74 = 51.75\ \mu g$ and the maximum amount per egg counted is:

$$\frac{51.75}{1.39 \times 10^8} = 37.2 \times 10^{-8}\ \mu g.$$

The amount expected in the polar bodies, assuming the haploid complement, is 36.4×10^{-8} μg (3×12.14), leaving 0.8×10^{-8} μg as the maximum amount that might be assigned to the egg cell. Thus taking all of the errors at their maxima, one can attribute to the egg cell an amount of DNA-thymine which cannot be more than 6 per cent of the amount in sperm. Perhaps it should be emphasized that the 6 per cent as determined above is not the amount that may be assumed to be present on the basis of probability but is the maximum amount obtained by calculations which set the quantities not at the means found but at the extreme of the range given by the standard errors of these determinations. In the calculations, sums, products and quotients of the standard errors were calculated by the usual formulae.

The finding of an amount of DNA equivalent to 6 N, where N represents the haploid quantity, in nuclei of the germinal vesicle stage of *Asterias* may be compared with the 4 N amounts obtained by microspectrophotometry determinations in young oocytes of the mouse (1), altho measurements in the germinal vesicle stage are not reported. The staining reactions of the pronuclei and polar bodies in *Tripneustes* are similar to those we previously reported for *Arbacia* and *Asterias* (23). Ludwig (16) has recently found that in the rat and in the hamster the polar bodies remain strongly Feulgen-positive thru the 3rd cleavage stage of the embryo. He also finds another condition analagous to that which we have described for echinoderms in

that the pronuclei of rodents go thru a stage before fusion where they become Feulgen and Methyl Green-negative. At the time this occurs the nucleus gives a positive Schiff reaction which the author suggests indicates a breakdown of DNA altho other interpretations are, without further evidence, equally plausible. It should also be mentioned that where measurements have been made in rodents and in annelids the first polar body has been found to have the diploid amount of DNA (2, 28).

Quite apart from the question of whether or not there is any DNA at all in the nucleus of the mature eggs of the species we have studied, there would seem to be no doubt from our results that large amounts of DNA or its derivatives are released to the cytoplasm during the maturation process. From evidence and arguments we will present shortly, it appears probable that the DNA products so released may act as inhibitors of the homologous ribose compounds known to be involved in the cell metabolism. Thus a non-uniform distribution of the desoxyribose compounds to different cytoplasmic sites would be equivalent to an unequal distribution of metabolic rate limiting factors and hence provide a clue to the origin of the different embryological potentialities of different regions of the egg cytoplasm. Large amounts of desoxyribose compounds in the cytoplasm of amphibian eggs have been reported by Hoff-Jørgensen and Zeuthen (13) altho in their case the origin of these compounds in sources other than the oocyte nucleus is not excluded. An interesting situation in non-gametic cells has been reported by Chayen and Norris (6) who found root meristem cells of *Vicia faba* with large cytoplasmic granules that seemed to contain most of the DNA of the cell. Unfortunately, neither of the abovementioned observations have a direct bearing upon the question of whether or not there is a phase in the nuclear cycle of the cells they investigated which is devoid of DNA.

In our experiments neither the isotope data nor the cytological observations alone are sufficient evidence for excluding DNA as the primary genic substance, but as we have tried to show, when both are taken together this is a necessary conclusion. Should it be assumed that the *Tripneustes* egg does contain an amount of DNA equal to about 6 per cent of the haploid amount, i.e. the amount indicated by taking the errors of our determinations at the maximum, obvious difficulties are encountered in applying the hypothesis that DNA is the genetic self-reproducing material. For example, 94 per cent of the haploid quantity of DNA must then be dispensible and consequently presumed to have a role other than that of self-reproduction as assumed by the hypothesis, which therefore becomes untenable unless additional assumptions are made that do not appear warranted.

If DNA is not an initiator of gene reactions, it may nevertheless function as a regulator of these reactions. A hypothesis postulating such action has been very briefly presented by one of us (20) in which it was assumed that the nuclear RNA is somehow involved in the synthesis of both those products which eventually give rise to the phenotypic expression of the gene as well as the synthesis of the new genic material itself. The hypothesis presupposes that there is sufficient similarity in the structure of DNA and RNA so that under appropriate conditions DNA may compete with RNA for enzymes or other elements in the metabolic systems in which they are involved. Evidence in support of this assumption is to be found in the observation previously reported that in the presence of bicarbonate buffer at pH 7.0 desoxyribonuclease will inhibit the rate of removal of RNA from the nucleus by nuclear enzymes (19). Another indication in the same direction is to be found in the observation that after removal of its purines, DNA is digested by ribonuclease (1). Since nuclear RNA is known to have a rapid turnover while DNA does not (8, 19, 30), it follows that DNA may act as an inhibitor of RNA. The hypothesis may be further generalized to include similar inhibitory action by desoxyribonucleotides and desoxyribonucleosides toward the corresponding ribose compounds. Experimental support for this generalization may be found in Carter's recent observation (5) that desoxyriboside 5′-phosphate inhibits oxygen consumption by mitochondria and that this inhibition is overcome by adenine ribose phosphate or polyphosphate.

There are a number of biological phenomena that do not fit the assumption that DNA is the genic material but which do fit the hypothesis we have proposed. There are others for which no adequate explanation exists that can be accounted for by this hypothesis. Some of these will be very briefly considered. Brachet (4) has made observations on hybrids between different species in the genus *Rana* which could not be explained by previous theories but can be accounted for by the present hypothesis. The hybrid embryos cease to develop after the end of the blastula stage at which time it is found that there is a marked diminution in the amount of DNA which can be detected cytologically in the nuclei; RNA turnover is enhanced over that found in control embryos while oxidations are diminished. Ordinarily the hybrid embryos die at this stage, but if the blastopore lip is transplanted to a normal embryo it proceeds to grow and differentiate. Brachet has pointed out that diffusible substances must be involved. If the observed deficiency in nuclear DNA is due to the release of unusual amounts of DNA derivatives to the cytoplasm, oxidations will be reduced by blocking of the ribonucleotides known to be involved and since a good portion of the DNA has been removed,

the nuclear RNA is capable of a more rapid turnover than normal. After transplantation further development of the transplant becomes possible either by the diffusion of the desoxyribose compounds out of the transplant into the normal host or by the diffusion into the transplant of ribose compound of the host or both.

A survey of investigations of reciprocal crosses shows that in plants the parental somatic effect is on the pollen while in animals it is on the egg. A correlation has been made between the presence of autolyzing tapetal cells in contact with the developing pollen in plants and the presence of autolyzing nurse cells and granulosa cells associated with developing eggs in insects and mammals (17, 18, 27, 29). Nutrional differences cannot account for the observations nor can the desoxyribonucleotide or nucleoside inhibitors here postulated be expected to exert an effect lasting more than one cell generation. However such a persisting effect can be accounted for by the transfer of more or less intact DNA, experimental evidence for which has been obtained in mammals (25).

The latter observations bear a resemblance to those made with the bacterial transforming substances and have already been suggested as a model for the mode of incorporation of the transforming DNA into the bacterial nucleus (25). The experiments with bacterial transforming substances are sometimes cited as evidence supporting the contention that genes are made of DNA altho a more cautious phraseology may be employed. Actually the experiments with the bacterial-transforming substances demonstrate that a directed change may be produced by the addition of DNA which persists for an indefinite number of cell generations and not that the DNA is the material which determines the type of primary reactions at any particular locus. The persisting transformation may be readily accounted for by our hypothesis. By intervening in the synthesis of the new chromosomal materials in a manner similar to that postulated for the gene products which are eventualy phenotypically expressed, the DNA creates a new condition at that site which is self perpetuating. The same kind of phenomenon is suggested also by the DS-AC modifiers of *Zea* described by McClintock (26) and the behavior of the "provirus" of lysogenic bacteria (15).

Altho results of experiments with *E. coli* bacteriophage are sometimes given as evidence that DNA is the primary genic material, the data cannot be taken as proof of this assumption, as has been pointed out by the authors of the papers cited (9, 10, 11). In relation to our hypothesis it is significant that before any new phage particles are formed in the first 10 minutes after infection, a quantity of new DNA of the phage type is synthesized which is

approximately equal to the amount of DNA originally present in the bacterium infected, this quantity being roughly about 60 times the amount in the infecting phage particle (12). In other words, very soon after the phage enters the cell a quantity of new DNA is synthesized which is adequate for the antagonism of the genic mechanism postulated by our hypothesis and this synthesis occurs in advance of and in excess of the need for actual incorporation into the phage particles. The theory also proposes that RNA be involved in the system in which DNA acts as an inhibitor. It is highly significant, therefore, that in this system, prior to the entry of P^{32} into the viral DNA, it is rapidly incorporated into a RNA which may be distinguished by its activity from the bulk of the cellular RNA present before infection, and that there is a continued synthesis and turnover of this RNA at a rate which is about the same as that for DNA formation (9). This phenomenon appears to be similar to one described for mammalian cells in mitosis where P^{32} is incorporated rapidly first into the nuclear RNA and subsequently into the DNA (19). The two situations, at least as far as the present data go, appear to be entirely analagous, the excess DNA formed on infection apparently being required for the regulation of the bacterial RNA under the new conditions.

The observations we have recorded in the present and the preceding paper on this subject demonstrate that in some species of eggs at the onset of ontology, DNA is either entirely absent or if it is there at all, it must be in amounts less than one twentieth of that found in the sperm. This observation makes the hypothesis that DNA is the sole or primary component of the gene untenable. The foregoing discussion of an alternative theory proposed earlier but given in more detail here does not constitute proof of that theory; but it indicates the generality of its application to apparently diverse biological phenomena, eliminates inconsistencies arising from other previous theories, and suggests methods for testing its validity experimentally. It is a theory that is obviously not complete; much remains to be elaborated, but in our opinion it does represent a departure in a new direction which promises to be fruitful in expanding our understanding of the mechanisms underlying biological growth, reduplication, and differentiation.

SUMMARY

1. Nuclei were isolated from *Asterias forbesii* oocytes in the germinal vesicle stage and the thymine content per nucleus found to be about six times the amount in the sperm of the same species.

2. Approximately the same yields of thymine were obtained by digesting sperm with 2 N $HClO_4$ under reflux for five hours and with 12 N $HClO_4$ for one hour.

3. The amount of thymine in the sperm of *Tripneustes ventricosus* was found to be 12.14×10^{-8} μg per cell.

4. The DNA-thymine content of a preparation of mature unfertilized *Tripneustes ventricosus* eggs was determined by the isotope dilution method using C^{14}-thymine of high specific activity in which all of the carbon atoms were uniformly labelled. All of the thymine found could be attributed to contaminating somatic nuclei, the number of which could be determined accurately, and to the polar bodies leaving none that could be assigned to the egg cells. Calculations made by setting the parameters not at the observed means but at the extremes of the range indicated by the standard errors gave the result that the amount of DNA-thymine in the egg could not be more than 6 per cent of the amont found in the sperm.

5. The nuclei of the mature unfertilized eggs of *Tripneustes*, like those of *Arbacia* and *Asterias*, gave a negative Feulgen reaction. After centrifugation at 78,000 g for 20 minutes nuclei of mature *Arbacia* eggs still gave a negative Feulgen reaction.

6. Reasons are given for concluding that DNA cannot be the primary genic material.

7. A hypothesis is presented giving the probable role of DNA and DNA-derivatives in the nucleus and the cytoplasm. A number of biological phenomena are interpreted in terms of this hypothesis.

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THE ULTRASTRUCTURE OF THE NUCLEAR MEMBRANE OF THE SEA URCHIN OOCYTE AS STUDIED WITH THE ELECTRON MICROSCOPE

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By utilizing the high resolution power of the electron microscope, the ultrastructure of the nuclear membrane was first revealed by Callan and Tomlin in 1950 (6), who worked with amphibian oocyte nuclei. The membranes of these giant nuclei were isolated, stretched on a supporting grid, washed and eventually metal-shadowed before microscopic examination. The electron micrographs revealed two layers in the nuclear membrane, an outer porous one consisting of normally tightly packed annuli, and an inner continuous one. Among later works those of Bovey (5), Bairati and Lehmann (3), and Gall (8), using the same technique on different material, are in agreement with Callan and Tomlin. Attempts to apply the replica technique to rat liver cell nuclei were not successful (4), although Callan and Tomlin have reported good results with amphibian oocyte material.

The ultra-thin sectioning technique has greatly increased the possibility of analysing the two layers and their relation to each other. In mammalian cells Anderson (1) reported finding no porous layer, Hartmann mentioned "radially oriented structural units" (11), but only Rhodin clearly demonstrated the presence of annular-like structures (15). The continuous layer has turned out to be bilaminar with a total thickness of around 200 Å units in most mammalian cell sections thin enough to permit this resolution (20, 7), similarly (11).

The ultrastructure of the nuclear membrane in invertebrate cells has also been investigated using the ultra-thin sectioning technique, Harris and James (10), and Bahr and Beermann (2).

Two modifications of the structure proposed by Callan and Tomlin may be mentioned. The nuclear membrane of the amoeba consists of the two layers mentioned, but the porous layer is directed inwards (3, 10). In some insect cells the nuclear membrane apparently consists only of a porous layer, with tightly or loosely packed annuli (5, 2).

It appears that the sea urchin oocyte offers a favorable subject for the study of the nuclear membrane. The first aim of this study has been to examine the connection between the two layers of the nuclear membrane.

MATERIAL AND METHODS

The sea urchins, *Echinus esculentus*, *Psammechinus miliaris*, *Strongylocentrotus droebachiensis*, and *Brissopsis lyrifera*, and the starfish, *Asterias rubens*, used in this investigation, were collected on the Swedish west coast. Oocytes from unripe females as well as mature eggs from ripe females were fixed in 1 per cent osmium-tetroxide dissolved in filtered sea water, in which the pH was approximately 8; this was sometimes adjusted to pH 7.2 with an acetate-veronal or phosphate buffer. The oocytes and eggs were then dehydrated and embedded in a mixture of *n*-butyl and methyl-methacrylate according to Newman, Borysko and Swerdlow (14). The sections were made with the ultrathin sectioning technique worked out by Sjöstrand (16, 18) using the microtome designed by him (17). A cutting edge of highly polished Schick razor blades was used. The sections were floated on a liquid surface (9) of 20 per cent ethyl alcohol.

The sections were floated onto the specimen grids covered with a thin formvar film. They were examined in the electron microsope without dissolving the methacrylate.

An RCA EMU 2c electron microscope was used with compensated standard objective pole piece, with standard objective aperture, with three apertures in the projector lens and reduced condensor lens aperture.

RESULTS

Porous layer.—The existence of a porous layer in the sea urchin oocyte is evident from sections cut obliquely (Fig. 1) or tangentially (Fig. 2) through the nuclear membrane. This layer appears as separated annuli. In the center of the annuli dark globules are often seen. The annuli often exhibit an elliptic rather than circular outline. This can be explained in two ways: (i) by oblique sectioning through a cylindrical figure, and (ii) by compression of the methacrylate during the sectioning. Deviations caused by these two phenomena have been calculated. Distortion caused by compression has been calculated to decrease measured values by as much as 20 per cent in the axis of sectioning. However, there may also be compensatory elongation in the perpendicular axis. Oblique sectioning of the annuli may increase measurement values by as much as 15 per cent. In order to avoid such errors straight tangential sections were used and measurements were made perpendicular to the sectioning direction. This latter direction has been estimated

by knife marks or from the form of the yolk granules and other spherical inclusions.

The mean value for the diameter of the annuli seen in the nuclear membrane of six *Echinus esculentus* eggs (as measured on nine photographs from three different animals) is 970 Å (965 ± 17 Å) in total. The ring wall measures 240 Å (242 ± 11 Å). The variations in the dimensions of the annuli in eggs of different sea urchins seem to be greater than the variations in the dimensions of the annuli in eggs of the same animal. The dimensions and appearance of the annulus are not changed by the different modifications of the fixation fluid.

In Fig. 2 some cracks may be observed following irregular courses between but never through the rings. Such cracks, probably caused during the fixation treatment, are more often observed in the nuclear membrane than in the surrounding cytoplasm.

Continuous layer.—To get a clear picture of a continuous layer in connection with the annuli it was necessary to orient the sectioning transverse to the nuclear membrane, which can be rather easily performed under a binocular microscope (magnification × 100).

In sections thus obtained the nuclear membrane appears as a clear-cut bilaminar structure (Fig. 3). The two osmium-impregnated single membranes, separated by a non-impregnated zone, do not follow an absolutely regular course. Some of the irregularities may be caused by distortion during the fixation. The inner single membrane often appears thicker and darker than the outer one. The dimensions for each have been calculated; the two mean values, however, do not show any significant difference. (See Table I.) When measuring the total thickness of the double membrane, places have been chosen in which the single constituents are parallel.

In sections, less than 300 Å thick, it can be seen that the nuclear membrane is not double throughout. Discontinuities, consisting of a "hole" covered by a single membrane apparently thinner than either of the two parts of the double membrane, are regularly found. They measure about 300–400 Å units in diameter. The outer and inner membranes are connected to each other at the sides of these discontinuities. It could be conjectured that these connections might give rise to the appearance of annuli in tangential sections of the double membrane. However, an encircling wall is found in connection with the discontinuity (Fig. 3); it projects approximately 600 Å on the nuclear side and 200 Å on the cytoplasmic side. These walls have a rather diffuse outline but have been measured and found to have dimensions which correspond well to those found for the annuli in tangential sections. The

TABLE I

Dimensions of the nuclear double membrane. All values are in Å units.

Total thickness of the double membrane	σ	Thickness of the inner single membrane	σ	Thickness of the outer single membrane	σ	Calculated height of the space between the single membranes	
315 ± 10^{1}	40	88 ± 3^{2}	11	84 ± 3^{2}	11	143	*Echinus esculentus*
343 ± 17^{3}	50	90 ± 5^{4}	10	88 ± 4^{4}	8	165	*Strongylocentrotus droebachiensis*
330 ± 32^{5}	71	91 ± 6^{5}	12	88 ± 5^{5}	12	151	*Brissopsis lyrifera*
306 ± 24^{6}	47	97 ± 7^{6}	14	92 ± 7^{6}	13	117	*Asterias rubens*
390 ± 34^{7}	76	90 ± 5^{8}	9	88 ± 5^{8}	9	212	*Psammechinus miliaris*, oocyte
329 ± 20^{9}	45	78 ± 6^{10}	14	79 ± 6^{10}	13	172	*Psammechinus miliaris*, ripe egg

[1] Calculated from 17 mean values* from 9 eggs of 4 animals
[2] ,, ,, 14 ,, ,, ,, 7 ,, ,, 4 ,,
[3] ,, ,, 9 ,, ,, ,, 2 ,, ,, 1 ,,
[4] ,, ,, 5 ,, ,, ,, 2 ,, ,, 1 ,,
[5] ,, ,, 5 ,, ,, ,, 4 ,, ,, 1 ,,
[6] ,, ,, 4 ,, ,, ,, 3 ,, ,, 1 ,,
[7] ,, ,, 5 ,, ,, ,, 2 ,, ,, 2 ,,
[8] ,, ,, 4 ,, ,, ,, 2 ,, ,, 2 ,,
[9] ,, ,, 7 ,, ,, ,, 3 ,, ,, 2 ,,
[10] ,, ,, 5 ,, ,, ,, 3 ,, ,, 2 ,,

* Each mean value obtained from *c.* 10 separate measurements.

space inside the ring seems to be filled with a loose gel, which holds the central globule in place. That this structure is a section through the long diameter of the annulus seems clear, since many transitions have been found after sectioning in other axes. In Text-Fig. 1 and Tables I and II the dimensions are given and in the diagrammatic Text-Fig. 2 all these details are shown.

In most cases the nuclear membrane exhibits a very irregular course with many large and small folds. The large ones orient themselves parallel to the inner membrane. Beside these folds, double membranes of the same thickness and with the same structure may be seen in the cytoplasm. These membranes appear to be discontinuous with, but close to the nuclear membrane. They are probably derived from the nuclear membrane and must not be confused with the "Golgi lamellae" or other cytoplasmic double

TABLE II

Dimensions of the nuclear annuli. All values are in Å units.

Inner height of the annulus	σ	Outer height of the annulus	σ	Total diameter of the annulus	σ	Thickness of the annular wall	σ	Diameter of the central globule	σ
				Echinus esculentus					
607±23[1]	77	210±13[1]	41	965±17[2]	49	242±11[2]	31	142±12[2]	36
				Strongylocentrotus droebachiensis					
604±42[4]	101	233±26[5]	58	1034±26[6]	52	216±26[6]	53	181±10[7]	15
				Brissopsis lyrifera					
606±128[8]	285	227±54[8]	107	896±15[9]	35	254±14[10]	28	166±13[8]	26
				Asterias rubens					
657±75[10]	149	166±33[10]	67						
				Psammechinus miliaris, oocyte					
706±154[11]	345	142±41[12]	71	757±27[12]	46	209±14[12]	25	149±1[12]	2
				Psammechinus miliaris, ripe egg					
502±42[13]	93	167±36[14]	72	1067±59[14]	117	304±28[14]	55	189±29[15]	51

[1] Calculated from 12 mean values* from 7 eggs of 4 animals
[2] ,, ,, 9 ,, ,, ,, 6 ,, ,, 3 ,,
[3] ,, ,, 6 ,, ,, ,, 5 ,, ,, 3 ,,
[4] ,, ,, 6 ,, ,, ,, 2 ,, ,, 1 ,,
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[8] ,, ,, 4 ,, ,, ,, 4 ,, ,, 1 ,,
[9] ,, ,, 6 ,, ,, ,, 3 ,, ,, 1 ,,
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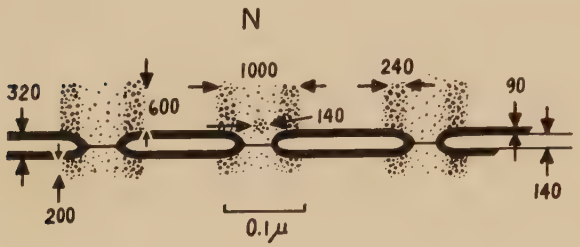
[11] ,, ,, 4 ,, ,, ,, 1 ,, ,, 1 ,,
[12] ,, ,, 3 ,, ,, ,, 1 ,, ,, 1 ,,
[13] ,, ,, 5 ,, ,, ,, 3 ,, ,, 2 ,,
[14] ,, ,, 4 ,, ,, ,, 2 ,, ,, 1 ,,
[15] ,, ,, 3 ,, ,, ,, 1 ,, ,, 1 ,,

* Each mean value obtained from *c.* 10 separate measurements.

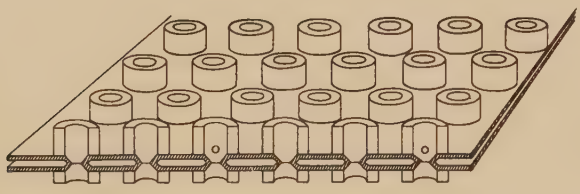
membrane systems, which for the sea urchin eggs and oocytes will be described in a following paper.

Remains of the oocyte nuclear membrane in the mature egg.—In the sea urchins, unlike the starfishes and most other animals, the maturation divisions are accomplished before fertilization. The diameter of the nucleus in the mature

egg is around 12 μ as compared with a diameter of 50 to 60 μ in the oocyte nucleus. (Measurements performed on living *Psammechinus miliaris* material.) Most or all of the oocyte nuclear membrane, therefore, must lose its function as a nuclear envelope. It does not, however, seem to undergo disintegration. In some eggs (most commonly *Psammechinus miliaris*) the cytoplasm contains a great number of unmistakable pieces of nuclear membrane.



Text fig. 1. Scheme of the nuclear membrane as seen in a transverse section. The dimensions are in Å units. *N* = nuclear side. Magnification 110,000 ×.



Text fig. 2. Scheme of the nuclear membrane. The cytoplasmic side is down. Magnification 60,000 ×.

See Fig. 4. For the most part, these have oriented themselves approximately parallel to each other and they can be found in loose aggregates of up to eight double membranes; thus there is a superficial resemblance to the Golgi system (7, 15, 19). The dimensions of the continuous layer and the annuli agree well with those measured in the oocyte. See also Tables I and II. Even in eggs 30 minutes after fertilization nuclear membrane fragments remain in the cytoplasm.

Nucleolus.—Whereas the nucleoplasm offers nothing of interest in the examined stages, the nucleolus of the oocyte may be briefly described. The diameter of the nucleolus may reach 16 μ; it stains readily with osmium tetroxide. No other structure has been detected than a mass of granules of about 300 Å in diameter and non-impregnated cavities of 0.1–1 μ. The larger of these cavities are also visible with light microscopy and after treatment with fixation fluids other than osmium-tetroxide. (See pictures in (13)). Besides the well-known large nucleolus smaller spheres of around 1.5 μ and of the same structure have been found.

DISCUSSION

In this paper the two layers of the nuclear membrane, originally described by Callan and Tomlin, have been called above by their original names, porous and continuous, although in the present case "annular" would be more expressive for the first and "discontinuous" for the second. The notion of two separate layers with different functions is also questionable. Callan and Tomlin definitely state that the porous layer could be washed away from the continuous one (in phosphate-ion containing solutions or mechanically at an air-water interface). However, this never seems to be a complete process; debris from the annuli always remain at the continuous membrane. At least for the sea urchin and starfish oocyte the two parts are so intimately connected that it is difficult to see how they could separate, and in fact they are united even when thrown out in the cytoplasm of the ripened egg. From a functional point of view it appears better to regard the system as a double membrane with discontinuities encircled by walls. The size of the discontinuites—around 300 to 400 Å—exceeds the size of any macromolecules—20 to 100 Å—which may be assumed to pass through the nuclear membrane. It is tempting to regard this structure as a mechanism for selective permeability. Arguments in favor of selective permeability of the nuclear membrane were raised by Anderson (1), and arguments against such selective permeability by Hogeboom and Schneider (12).

It is a remarkable fact that an annular organisation in the nuclear membrane has been found in widely different groups in the animal kingdom. Perhaps this points to a general principle for nuclei in an active phase. The annuli may be very difficult to discern on sections through the membrane, especially when the fixation has left many substances of similar density close to the membrane. Obliquely cut and very thin sections are best fitted for the first tracing.

The nuclear membrane often appears to be in excess in the sea urchin oocyte giving the nucleus a wrinkled appearance. This may be due to fixation artefact but may also be lifelike and thus explain the well-known ability of the nucleus to swell. The membranes lying outside the nuclear membrane, with the same structure as this, show no connection with the nucleus. Similarly, extra-nuclear membranes are reported by Dalton and Felix (7) in epithelial cells of the mouse epididymis. The function of these accessory membranes of the oocyte, and of the orderly arranged membrane remnants of the ripe egg, remains obscure.

SUMMARY

The ultrastructure of the nuclear membrane from sea urchin and starfish oocytes has been studied by means of electron microscopy in ultra-thin sections prepared according to the technique of Sjöstrand.

1. The nuclear membrane appears as a double membrane with 40 to 80 "holes" per μ^2. These "holes" are covered with a very thin single membrane and are surrounded by encircling walls. The latter correspond to the annuli described by earlier authors.

The total dimension of an annulus is around 1000 Å, with a thickness of the annular wall 200–250 Å. Its inner height is 600 Å and the outer 150–250 Å. The double membrane measures 300–400 Å with the single constituents measuring around 90 Å.

2. An irregular extra-nuclear membrane with the same structure as that described in point 1, above, is often found in the sea urchin oocyte.

3. During maturation divisions the nuclear membrane breaks and the remnants become arranged in parallel order in the ripe egg.

The author wishes to thank Dr. F. Sjöstrand, Associate Professor of the Department of Anatomy, for the privilege of working in his laboratory and for many stimulating discussions. He is also grateful to his teacher Professor J. Runnström for helpful criticism and continued interest, and to Dr. G. Gustafsson, Kristineberg, for generous help and supply of material.

He also wishes to record his thanks to Dr. J. Rhodin for instructing him in the use of the electron microscope, to Miss Maj Berghman for valuable help with the figures, and to Dr. H. Sheldon for correcting the translation.

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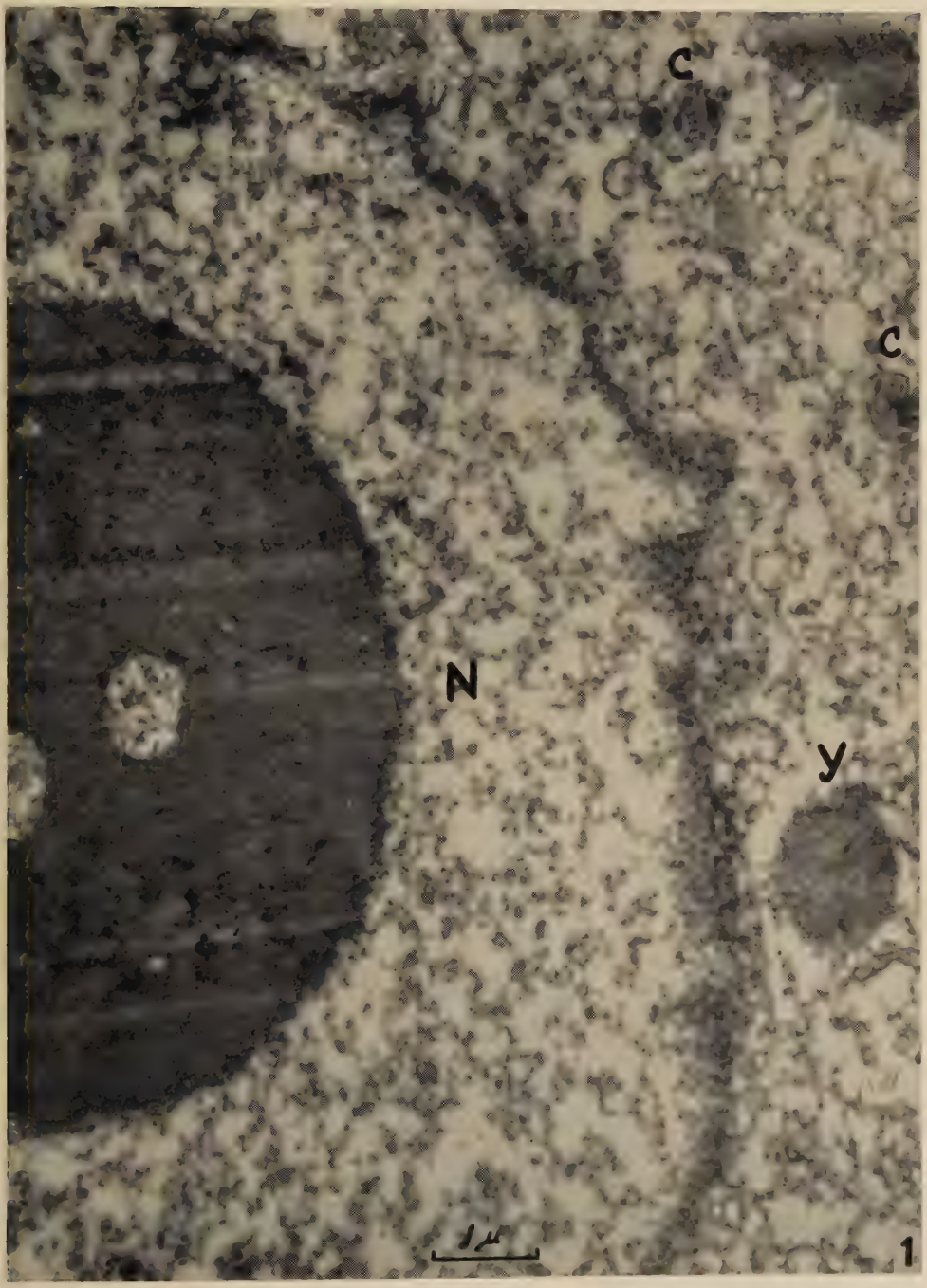
Fig. 1. *Echinus esculentus* oocyte. The nucleus, *N*, with the dark nucleolus to the left. In the obliquely cut nuclear membrane annuli are to be seen. *C* = cortical granule, *Y* = yolk granule. Magnification 15,000 ×.


C
C
N
Y
1μ
1

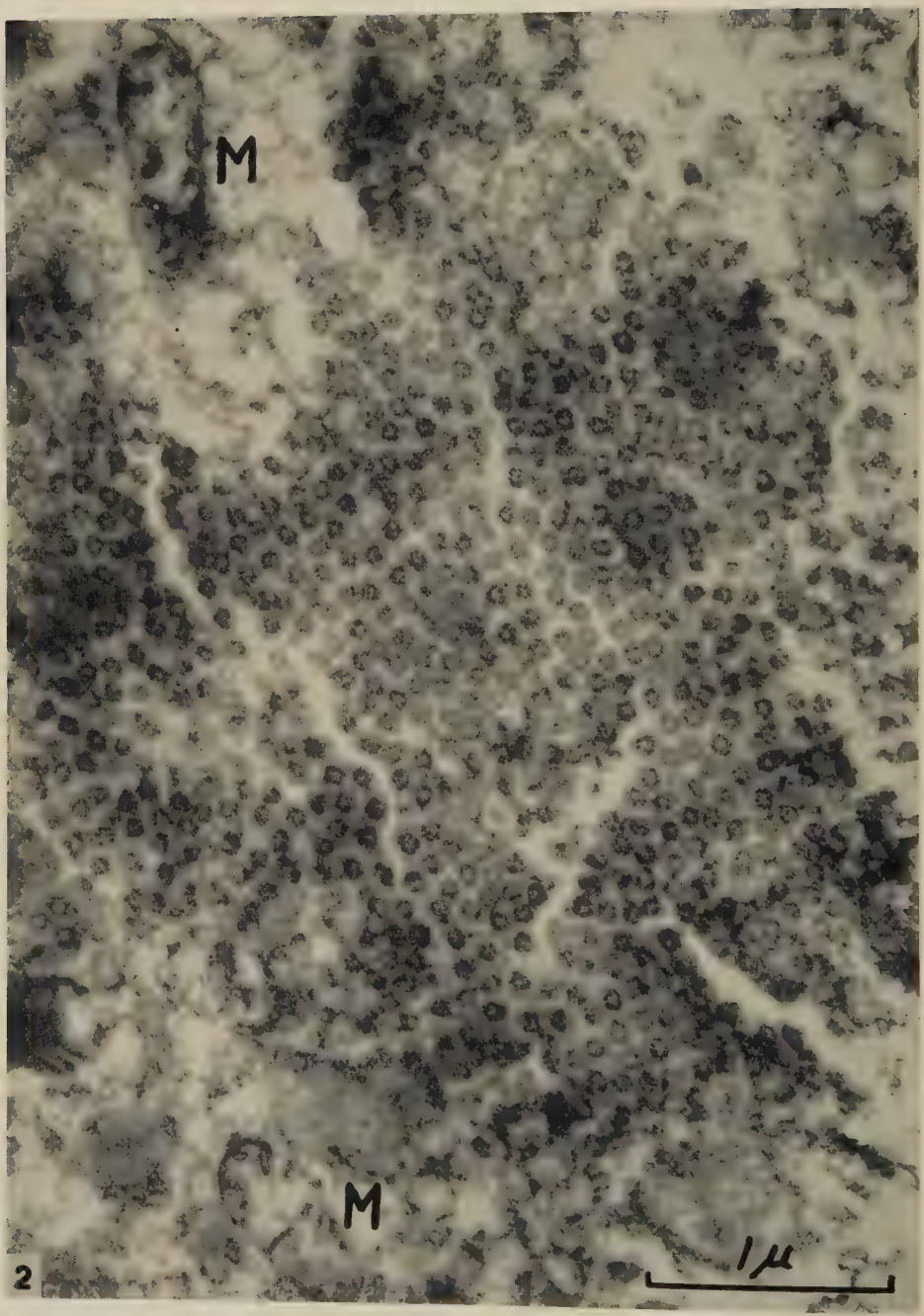


Fig. 2. *Brissopsis lyrifera* oocyte. The nuclear membrane tangentially sectioned. *M* = mitochondrion. Magnification 32,000 ×.

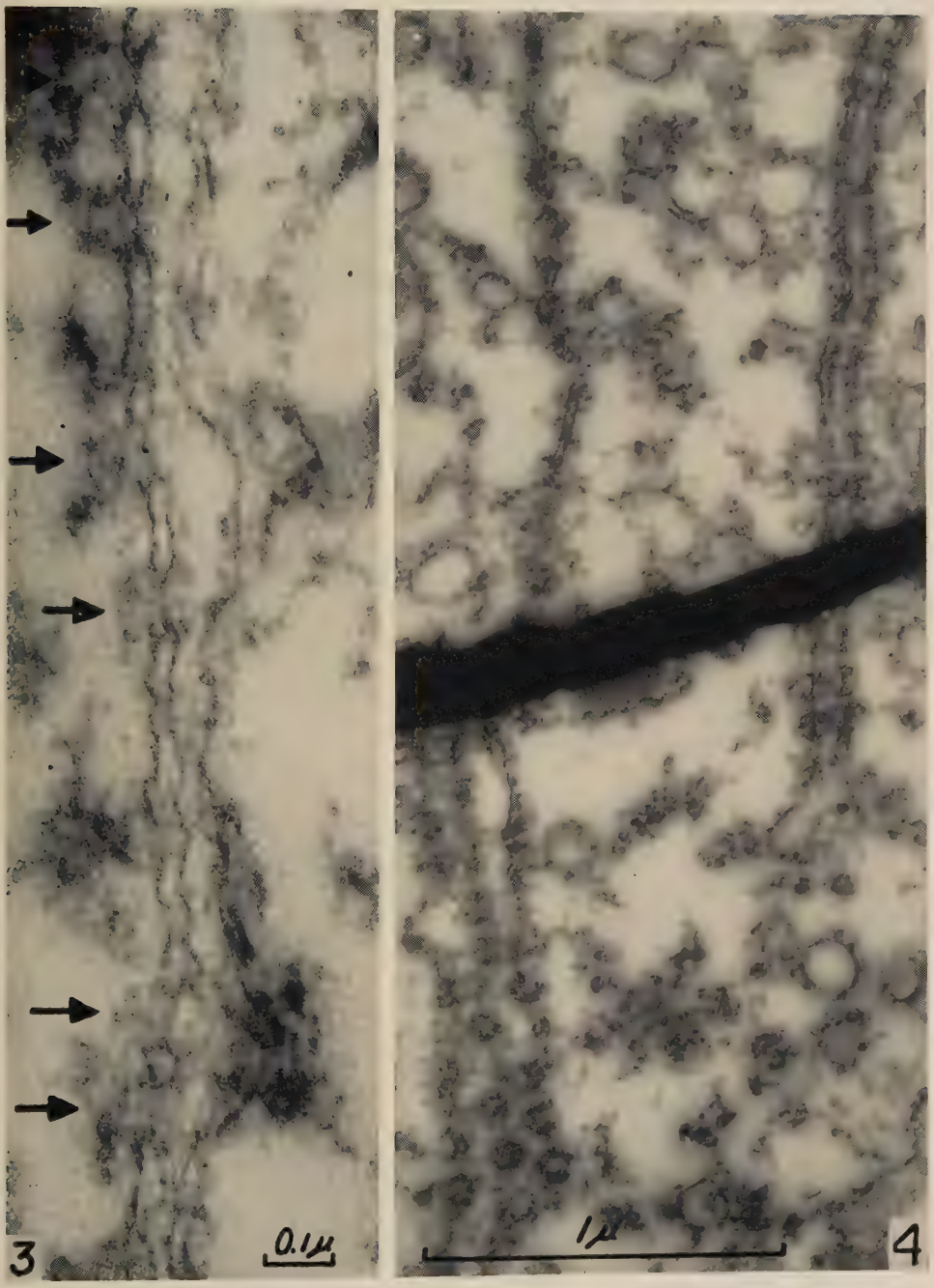


Fig. 3. *Echinus esculentus* oocyte. The nuclear membrane tranversely sectioned. The arrows in the nuclear side show six annuli. Magnification 95,000 ×.
Fig. 4. *Psammechinus miliaris*, ripe egg. Aggregate of nuclear membranes. The dark band is caused by a fold in the section. Magnification 49,000 ×.

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HISTOCHEMICAL DEMONSTRATION OF SITES OF ACID PHOSPHATASE ACTIVITY IN MITOCHONDRIA[1]

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Biochemical studies made on rat liver homogenates have shown that acid phosphatase is principally a cytoplasmic enzyme, the largest portion being located in the mitochondrial fraction (1, 6, 7). The reports of histochemical localization of this enzyme, based on precipitation of lead phosphate, have been contradictory to the biochemical data. With histochemical methods, little cytoplasmic activity but heavy nuclear precipitates have been shown in the rat liver (7, 8, 9), monkey liver (2), and fibroblasts (5). Goetsch and Reynolds (3), however, have found sites of activity to be in cytoplasmic structures of the liver and the renal tubule.

In the course of histochemical studies on the digestive organs of the rainbow trout, *Salmo gairdnerii irideus*, sites of acid phosphatase activity were determined. The same test was applied to the rat liver.

METHOD

The tissue was fixed in cold acetone for 24 hours at 4° C, followed by dehydration in absolute acetone under the same conditions. It was then cleared, at room temperature, in two changes of benzene, 45 minutes each, infiltrated in three changes of 53° - 55° C paraffin, 30 minutes each, and paraffin embedded at 54° C. Chilled blocks were cut at 6 μ, the tissue floated on the slide with distilled water, using no albumin-glycerin or other adhesive (3), and dried on a 37° C heating table. Further drying was accomplished overnight in a 37° C incubator.

The incubating medium and procedure that followed is essentially that of Gomori (4). The stock medium was buffered with 1 M acetate to pH 4.9 and diluted with two parts water. The water used in all solutions was prepared by demineralizing ordinary distilled water by passing it through an ion exchange resin filter (Deeminite, Crystal Research Laboratories, Inc., Hartford, Conn.). The slides were incubated for eight hours at 37° C, without removal of paraffin as recommended by Goetsch and Reynolds (3). Excess precipitate was removed by rinsing in 2 per cent acetic acid. Control slides were incubated simultaneously, in which case water replaced the substrate in the medium. No counterstain was used.

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Consecutive sections were stained with Harris' hematoxylin and eosin, and 0.1 per cent toluidine blue in citrate buffer. Control slides were pretreated with 10 per cent perchloric acid for 18 hours at 4° C to remove ribonucleic acid.

DISCUSSION

In the epithelial cells of the trout cardiac stomach and intestine the sites of acid phosphatase activity were predominantly in the cytoplasm with only minimal precipitation in the nucleus. In the cytoplasm the enzyme was visualized in the mitochondria with varying amounts of diffusion into the surrounding regions. Corresponding structures stained basophilic with hematoxylin and toluidine blue; however, loss in staining intensity followed incubation in perchloric acid. Therefore, some RNA as well as acid protein was preserved in the mitochondria.

Since this technic proved satisfactory with trout tissues, it was applied to the rat liver, the tissue usually used for acid phosphatase studies. The localization of enzyme activity was the same. The mitochondria, though small, were distinctly visible as black rods, with diffusion into the cytoplasm. A heavy precipitate was present along the bile canaliculi.

Optimal results, showing distinct mitochondria, are obtained only when fixation, clearing, embedding, cutting, and incubation of the tissue follow in rapid succession. Storage, even at low temperatures, causes loss of enzyme activity and inferior results. Rapid drying in the incubator proved to be more satisfactory than the recommended air drying (3).

The precipitate in the mitochondria is not homogenous, but assumes an alternately dark and light pattern. These differences in intensity indicate varying concentrations of enzyme, and are probably due to lack of preservation of the mitochondrial framework with consequent irregular precipitation of the enzyme during fixation. Within a few hours after visualization of the lead phosphate as a sulphide, the coloration fades more rapidly in the areas of lesser concentration intensifying the pattern in the rod, which finally appears as granules. The granulation is maximal after 12 hours and

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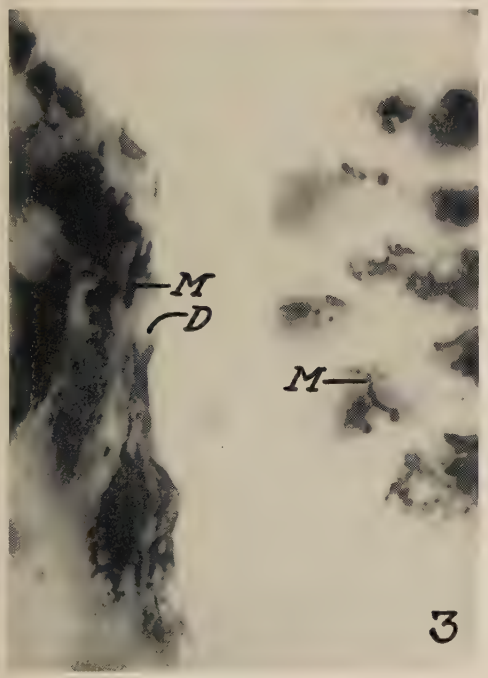
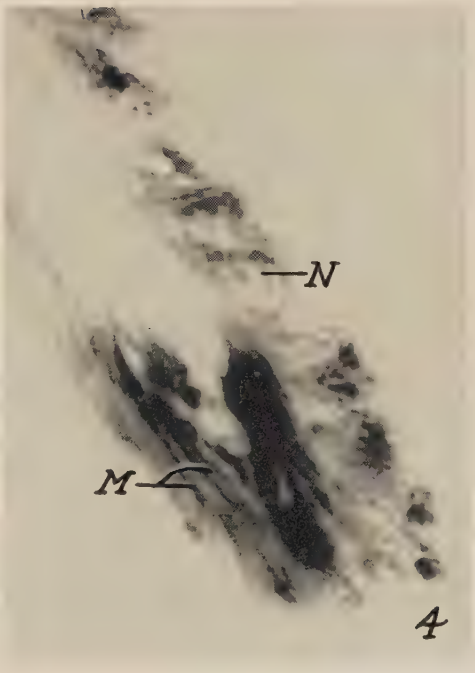
The photomicrographs were made with the Leica and Leitz Micro-ibso attachment, Wratten filter No. 58, and 100 watt Zirconium arc lamp. Tissue in all figures is trout cardiac stomach. Magnification × 1350.

Fig. 1. Cardiac gland cells. *M.*, mitochondria.
Fig. 2. Control section of gland cells.
Fig. 3. Cardiac gland cells. *M.*, mitochondria; *D.*, diffusion.
Fig. 4. Columnar cells. *M.*, mitochondria; *N.*, nucleus.

M
1

2


M
D
M
3


N
M
4

after 24 hours few rods are left. The fading is partially attributed to the mounting medium.

The contradictory results previously reported have probably been due to loss of sites of activity by diffusion resulting in nuclear artefacts. The modifications given and those of Goetsch and Reynolds (3) tend to minimize such effects.

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OBSERVATIONS ON NUCLEAR SIZE AND FORM IN LIVING RAT AND MOUSE EGGS

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Received July 2, 1954

FIGURES have already been reported for the volumes of pronuclei and nuclei, for the total volumes of nucleoli, and for the numbers of nucleoli in rat eggs at various stages in the course of normal fertilization (3) and in early cleavage (4). Similar data have also been given for 2-cell rat eggs that were polyspermic and almost certainly triploid (5). Information has now been gained on the pronuclei of polyspermic rat eggs and also on the nuclei in 1-cell and 2-cell rat and mouse eggs that had been activated artificially and were evidently haploid (6, 10). Thus, nuclear size and form in eggs that may reasonably be supposed to have had haploid, diploid, and triploid chromosome complements have been compared, and the results are the subject of the present communication.

METHODS

To obtain polyspermic eggs, female rats were permitted to mate after the time of ovulation, a procedure that ensures a relatively high incidence of polyspermy (5). Rats with copulation plugs were killed 18–20 hr. after ovulation, when pronuclear development is maximal (3), and the eggs were examined under the phase-contrast microscope (Fig. 2). Pronuclei and nucleoli were measured with an eyepiece micrometer, as previously described (3).

Apparent early parthenogenetic development was induced in the eggs of unmated rats as described elsewhere (6); it was recognised by extrusion of the second polar body and by the formation of a single large nucleus in the undivided egg (Fig. 3), and, in later stages, by cleavage into 2-cell eggs that closely resembled the corresponding stage in fertilized eggs (Fig. 5). Calculations of nuclear and total nucleolar volumes were made in the same way as for the pronuclei in normal and polyspermic eggs.

Female mice were caged with males and examined each morning for evidence of mating. Those with copulation plugs were killed between 4.0 p.m. and 9.0 p.m. the same day, or on the following morning, between 10.0 a.m. and mid-day. Mice

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killed between 4.0 p.m. and 9.0 p.m. provided eggs in the course of fertilization; the pronuclei (Fig. 6) were considered to be fully developed, because, in several instances, eggs recovered between 8.0 and 9.0 p.m. showed stages of syngamy or had actually undergone the first cleavage. The 2-cell eggs required for study (Fig. 8) were obtained from the mice killed on the morning of the day after the finding of the copulation plug. Determination of nuclear and nucleolar volumes was made in the same way as for the rat eggs.

Mouse eggs were artificially activated by applying hot-shock treatment about mid-day to mice in which ovulation had occurred during the previous night (10). The mice were killed between 9.0 a.m. and 10.0 a.m. the following morning and measurements were made on undivided eggs that had developed single nuclei (Fig. 7) and on 2-cell eggs that had no recognisable polar body (Fig. 9). Evidence previously submitted (10) indicates that these 2-cell eggs were probably formed, less than 4 hours after the application of hot-shock, by a process referred to as "immediate cleavage". From considerations of their mode of formation in which the second meiosis is apparently associated with cleavage instead of polar-body extrusion and from determinations of chromosome number, it is evident that the nuclei of such eggs are haploid and actually correspond to female pronuclei.

RESULTS

Nuclear size and form in rat and mouse eggs.—The mean figures obtained for the nuclear and total nucleolar volumes, and for the numbers of nucleoli, are shown in Tables I and II, together with the range and the standard error of the mean.

Some of the comparisons made in this paper were necessarily based upon tests of statistical significance; the relevant figures are as follows: the mean total nucleolar volume of the mononucleate undivided rat eggs (620.4 cu.μ) is significantly less ($P < 0.001$) than the mean of the sums of the nucleolar volumes of normal male and female pronuclei (772.1 cu.μ). The mean nuclear volume of the induced 2-cell rat eggs (2141.5 cu.μ) is significantly less ($P < 0.01$) than the volume in normally fertilized 2-cell eggs (2808.3 cu.μ). The mean nuclear volume of mononucleate undivided mouse eggs (4083.9 cu.μ) is significantly less ($P < 0.05$) than the mean of the sums of the male and female pronuclear volumes for each normal egg (5792.8 cu.μ). The mean total nucleolar volume of mononucleate undivided mouse eggs (435.3 cu.μ) is significantly less ($P < 0.001$) than the mean of the sums of nucleolar volumes of male and female pronuclei for each egg (500.1 cu.μ).

One rat egg that was observed, but not included in Table I, was of "giant" proportions, similar to those described elsewhere (7), except that it had an even larger volume—approximately 2½ times the normal. This egg had been

Description of eggs	Number of eggs	Mean nuclear volume cu.μ		Mean total nucleolar volume per nucleus cu.μ		Mean number of nucleoli per nucleus	
		♂ Pronuclei	♀ Pronuclei	♂ Pronuclei	♀ Pronuclei	♂ Pronuclei	♀ Pronuclei
Fertilization—normal[1]	17	5702.7±161.04[2] (range 4340–6842)	2314.2±66.19[2] (range 1764–2810)	550.9±13.56[2] (range 481–662)	221.2±7.94[2] (range 170–276)	17.1 (range 5–36)	9.0 (range 5–12)
Fertilization—polyspermy (dispermy)	11	3189.7±91.75 (range 2345–4130)	962.0±67.27 (range 618–1304)	334.6±8.94 (range 248–422)	123.8±6.34 (range 84–153)	10.7 (range 5–20)	4.3 (range 1–8)
Artifical induction, single nucleus	7	—	4188.1±439.03 (range 2165–5553)	—	620.4±27.42 (range 549–721)	—	9.6 (range 5–19)
2-cell—normal[1]	40	2808.3±42.11 (range 1867–3630)		323.5±5.97 (range 241–616)		5.4 (range 2–11)	
2-cell—polyspermy[1]	7	2930.9±158.81 (range 2108–4158)		328.1±11.88 (range 277–422)		5.9 (range 2–10)	
2-cell—artificial induction	15	2141.5±71.55 (range 1425–2785)		306.0±10.34 (range 195–412)		7.3 (range 1–15)	

[1] Data reported previously (3, 4, 5). [2] Standard error.

TABLE II

Volumes of nuclei and nucleoli, and number of nucleoli, in mouse eggs.

Description of eggs	Number of eggs	Mean nuclear volume cu.μ		Mean total nucleolar volume per nucleus cu.μ		Mean number of nucleoli per nucleus	
		♂ Pronuclei	♀ Pronuclei	♂ Pronuclei	♀ Pronuclei	♂ Pronuclei	♀ Pronuclei
Fertilization	10	4112.2±141.01[1] (range 3442–4770)	1680.6±103.24[1] (range 1349–2432)	324.4±8.19[1] (range 296–384)	174.7±4.82[1] (range 155–195)	5.0 (range 1–7)	2.3 (range 1–5)
Artifical induction, single nucleus	8	—	4083.9±384.22 (range 2325–5520)	—	435.3±13.30 (range 366–487)	—	11.9 (range 5–18)
2-cell—normal	10	2157.0±285.38 (range 1697–2885)		253.3±6.82 (range 206–301)		10.4 (range 3–20)	
2-cell—artificial induction ("Immediate cleavage")	5	2323.2±167.67 (range 1570–3200)		255.0±15.34 (range 204–357)		4.5 (range 2–7)	

[1] Standard error.

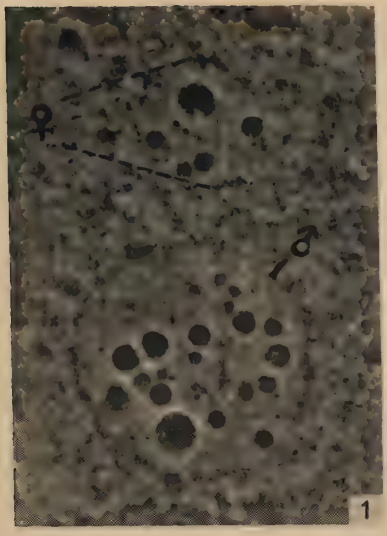
subjected to cold-shock treatment *in vivo*; the volume (4990 cu.μ) of its nucleus (Fig. 4) differed insignificantly from the mean volume of the induced nuclei in the eggs of normal size (4188.1 cu.μ), but the total nucleolar volume (1258 cu.μ) was about twice the mean for the other mononucleate eggs (620.4 cu.μ).

Vitelline volume of rat and mouse eggs during fertilization.—The mean volume of the vitellus of sperm-penetrated rat eggs in the fresh state was reported to be 195,000 cu.μ (7). Measurements have now been made on mouse eggs under the same conditions. The mean diameter of 25 eggs was 73.4 μ (range 70.3 μ to 77.8 μ). This mean value corresponds to a volume of 207,000 cu.μ, which does not differ significantly from the volume determined for the rat eggs.

DISCUSSION

The genesis of male and female pronuclei has been observed in living rat eggs (1). The early male pronucleus becomes recognisable with the formation of a number of small nucleoli in the structure representing the enlarged and modified sperm head. The female pronucleus, on the other hand, is first evident following the formation of nucleoli in the compacted group of chromosomes that remain within the egg after the abstriction of the second polar body. Thus the male pronucleus in the rat egg is, from its inception, a distinctly larger structure than the female pronucleus. The genesis of the pronuclei is evidently dependent upon some property of the vitelline cytoplasm which is not always shared by the polar-body cytoplasm. This is shown by the behaviour of the group of chromosomes that passes into the second polar body; the group is presumably identical, except in genetic characters, to that from which the female pronucleus arises, yet it often fails to form a nucleus. The emergence of the pronuclei is closely synchronous; seldom does one pronucleus appear before the other, even if the correlation of the preceding stages has been disturbed experimentally (2, 8). Throughout later development, also, the pronuclei grow at the same relative rate, for in the rat the male pronucleus always has about twice the volume, and twice the nucleolar volume and number, as the female (3).

Fig. 1. Normal pronuclei at full development in rat egg. × 950.
Fig. 2. Pronuclei at full development in rat egg undergoing polyspermic fertilization. × 950.
Fig. 3. Nucleus found in unfertilized rat egg after cold-shock treatment. This egg was of normal size. × 950.
Fig. 4. Nucleus found in unfertilized rat "giant" egg after cold-shock treatment. × 950.
Fig. 5. Nuclei of 2-cell unfertilized rat egg recovered after cold-shock treatment. The division between the blastomeres runs horizontally across the picture. × 950.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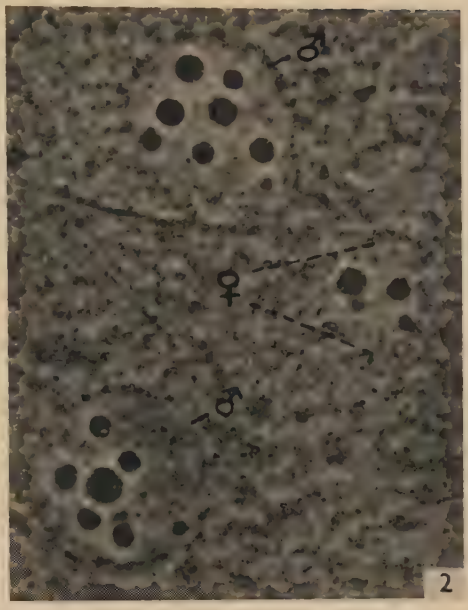
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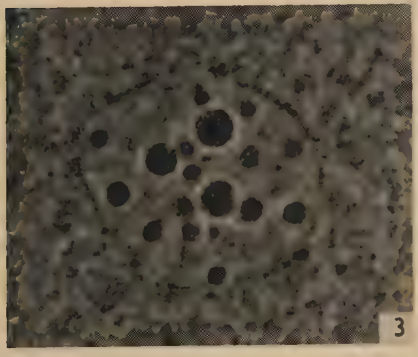
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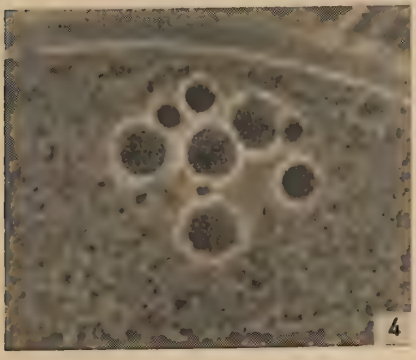
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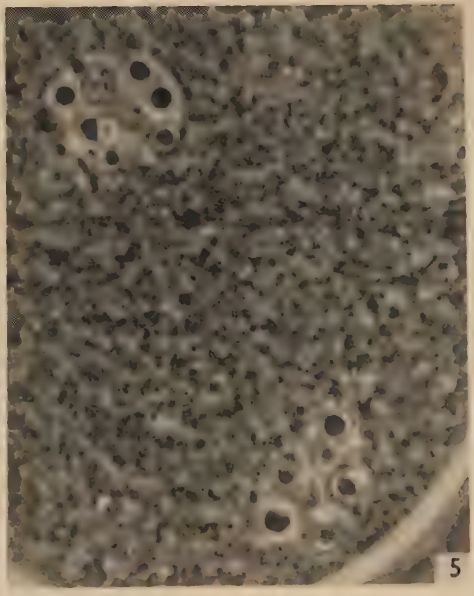
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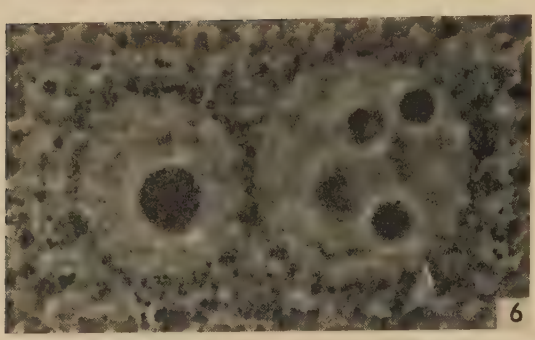

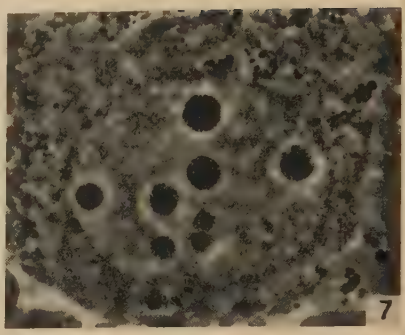

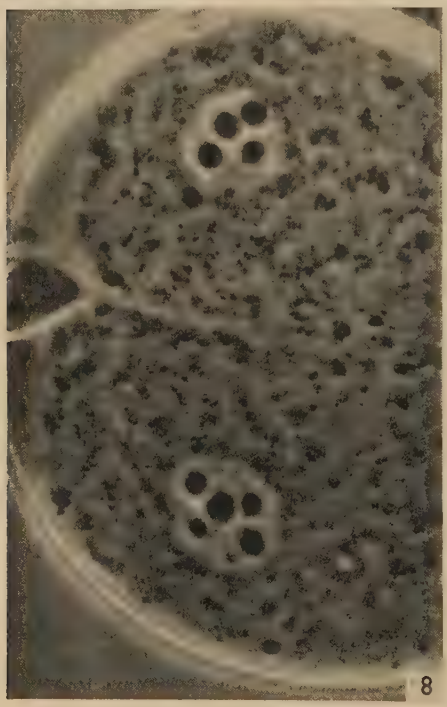

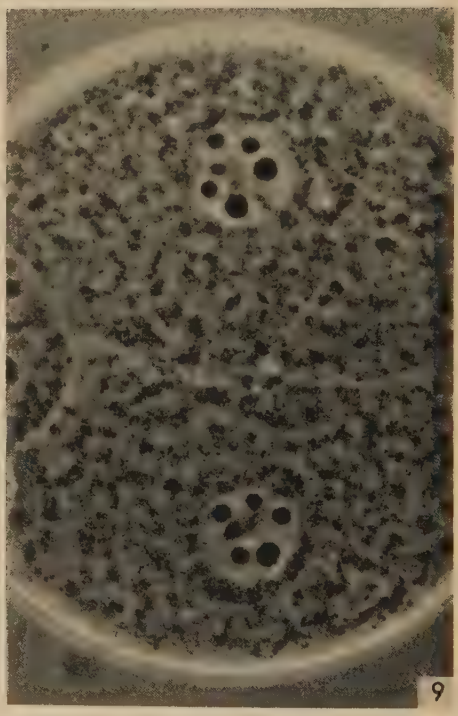

Fig. 6. Normal pronuclei at full development in mouse egg. × 1300.
Fig, 7. Nucleus found in unfertilized mouse egg after hot-shock treatment. × 1300.
Fig. 8. Nuclei in 2-cell fertilized mouse egg. × 800.
Fig. 9. Nuclei in unfertilized 2-cell mouse egg after hot-shock treatment. This egg was probably formed by the process referred to as "immediate cleavage". × 800.

For the purposes of pronuclear growth, material is presumably drawn from cytoplasmic stores in the egg, and because the male pronucleus is about twice the size of the female, at least in the rat and mouse, it would

seem that the male has a greater affinity for the material. This suggests that a competition may exist between the pronuclei for the substances required for growth and raises the possibility that competition is one of the factors that determine the ultimate size achieved by the pronuclei. Evidence for such a hypothesis is found in observations made on polyspermic rat eggs. When two male pronuclei and a female pronucleus develop in an egg neither of the male pronuclei acquires the size of the male pronucleus in a monospermic egg and the dimensions of the female pronucleus are greatly reduced. Indeed, the sum of the nuclear volumes (7341.4 cu.μ), and also the sum of the total nucleolar volumes (793.0 cu.μ), in polyspermic rat eggs do not significantly exceed the corresponding values for monospermic eggs (8016.9 cu.μ and 772.1 cu.μ, respectively). This must mean that the vitelline constituents for which the pronuclei compete are available in quantities no greater than actually required for the development of normal pronuclei.

In both rat and mouse eggs, the formation of a single nucleus can be induced by artificial stimuli: cold-shock for the rat egg, and hot-shock for the mouse egg. The single nucleus represents a female pronucleus developing without competition from a male pronucleus and it is notable that, under these circumstances, both nuclear and total nucleolar volumes are much larger than those of a female pronucleus in an egg undergoing normal fertilization. However, the single nucleus is significantly smaller in respect of both nuclear and nucleolar volumes than the male and female pronuclei taken together. These facts further support the idea of competition between the pronuclei, but also indicate that the availability of cytoplasmic materials is not alone responsible for limiting the size of pronuclei. It seems that some limitation to size is inherent in the pronuclei themselves. The innate restriction on nuclear volume is more evident than that on total nucleolar volume, particularly in rat eggs. This difference is well shown by the "giant" egg that was found to have an induced nucleus; although the vitellus was about $2\frac{1}{2}$ times the size of a normal egg, the nuclear volume (4990 cu.μ) was virtually the same as in the other induced nuclei (mean, 4188.1 cu.μ). On the other hand, the total nucleolar volume (1258 cu.μ) was about twice that in the other induced nuclei (mean, 620.4 cu.μ). The existence of a larger source of cytoplasmic materials apparently enhanced only nucleolar growth.

Mouse eggs exhibiting "immediate cleavage" are of special interest, for it would seem certain that their nuclei are the products of the second meiotic division. If this is true, the nuclei represent pronuclei that have developed in

half the normal amount of cytoplasm. Consistently, the nuclei were found to have about half the nuclear volume and half the total nucleolar volume of the induced nuclei in undivided eggs. The mean number of nucleoli was also half the mean number in the undivided eggs.

The conclusions to be drawn so far may be briefly stated as follows: Pronuclear growth depends upon the utilization of specific material present in the egg, and the pronuclei compete for this material, the male pronucleus, in the rat and mouse, having a greater affinity for it than the female pronucleus. Competition is evidently responsible for the development of smaller pronuclei in polyspermic eggs, and this also implies that the limited availability of the materials required for pronuclear growth is a normal controlling influence on pronuclear size. Even when nuclear development has occurred in the absence of competition, however, the size is smaller than theoretically possible, so that there must also be an inherent limitation to size. The innate restriction on nuclear volume is more severe than that on total nucleolar volume.

The number of nucleoli per nucleus varies during the course of pronuclear growth in the rat egg (3), but the mean number seen at full development seems to be chiefly related to nuclear size. Thus, in both rats and mice, there are twice as many nucleoli in the male pronuclei as there are in the female. Moreover, in polyspermic rat eggs, the nuclei have fewer nucleoli and the mean number contained in the three pronuclei of these eggs is about the same as the mean number in the two pronuclei of monospermic eggs. Divergence from this principle is seen in the induced nuclei which contain, relative to nuclear volume, fewer nucleoli in rat eggs, and more nucleoli in mouse eggs.

It might be expected that the mean volume of the nuclei of normally fertilized 2-cell eggs would be one-half the sum of the pronuclear volumes. In both rat and mouse eggs, however, the volume of the 2-cell nucleus is less than this. On the other hand, the mean total nucleolar volumes more closely approach the 2:1 relationship, particularly in the mouse egg. Ploidy has little influence on nuclear size and form, for polyspermic 2-cell rat eggs, which have been shown to be almost certainly triploid, do not differ significantly, either in nuclear volume, total nucleolar volume or nucleolar number, from normal diploid eggs (5). Unfertilized 2-cell rat eggs found after hot-shock treatment are almost certainly haploid (6), yet the mean total nucleolar volume and nucleolar number of their nuclei does not differ significantly from corresponding values in diploid eggs, and the mean nuclear volume is not much less than that of diploid eggs. The virtual independence

of ploidy that is exhibited by the 2-cell nuclei, in respect of their size and form, is evidently peculiar to eggs, for the characteristics of somatic nuclei in many invertebrate and vertebrate species have been said to reflect variations in chromosome number (9, 11).

From the measurements made it is apparent that rat and mouse eggs have approximately the same vitelline volume, namely, about 200,000 cu.μ. The nuclear volumes and the total nucleolar volumes of male and female pronuclei and of normal 2-cell nuclei, are all 1.3 to 1.7 times larger in rat eggs than the corresponding volumes in mouse eggs. It seems logical, however, to suggest that the nucleo-cytoplasmic ratio is really the same in the eggs of both species and that there is a larger proportion of yolk in the mouse egg. Certainly, the mouse egg has a more coarsely granular cytoplasm than the rat egg and, in the authors' experience, is more prone to apparent deutoplasmolysis.

SUMMARY

Figures are presented for the mean nuclear volume, the mean total nucleolar volume, and the mean number of nucleoli, in the pronuclei of polyspermic rat eggs, in the nuclei of artificially activated 1-cell and 2-cell rat eggs, in the pronuclei and 2-cell nuclei of fertilized mouse eggs, and in the nuclei of artificially activated 1-cell and 2-cell mouse eggs. From a consideration of these data, and of information previously published, the following conclusions have been reached for the eggs of rats and mice:

1. To fulfil the requirements of pronuclear growth, material is drawn from cytoplasmic stores in the egg and is available in just sufficient quantity for the development of pronuclei of normal size and number. Limited availability of material thus constitutes a normal restriction to the growth of the pronuclei.

2. The pronuclei compete for the formative material and the male pronucleus appears to have the greater affinity for it, since it maintains about twice the size of the female pronucleus throughout development.

3. There is, at least in the female pronucleus, also an inherent limit to size; this is not normally operative but becomes so when a female pronucleus develops in the absence of competition from a male. The restriction affects nuclear volume more than total nucleolar volume.

4. Nuclear volume and total nucleolar volume in 2-cell eggs is apparently unrelated to ploidy, since haploid, diploid and triploid eggs have nuclei that differ little from each other. Similarly, the number of nucleoli is evidently no guide to chromosome number.

5. Although mouse and rat eggs have the same vitelline volume (about 200,000 cu.μ), the pronuclei and nuclei are smaller in the mouse egg. It seems likely, however, that the nucleo-cytoplasmic ratio is in reality the same for both species and that the apparent difference in ratio should be imputed to the presence of more yolk in the mouse egg.

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CAFFEINE INHIBITION OF FERTILIZATION IN *ARBACIA*[1]

ELECTRON MICROSCOPY

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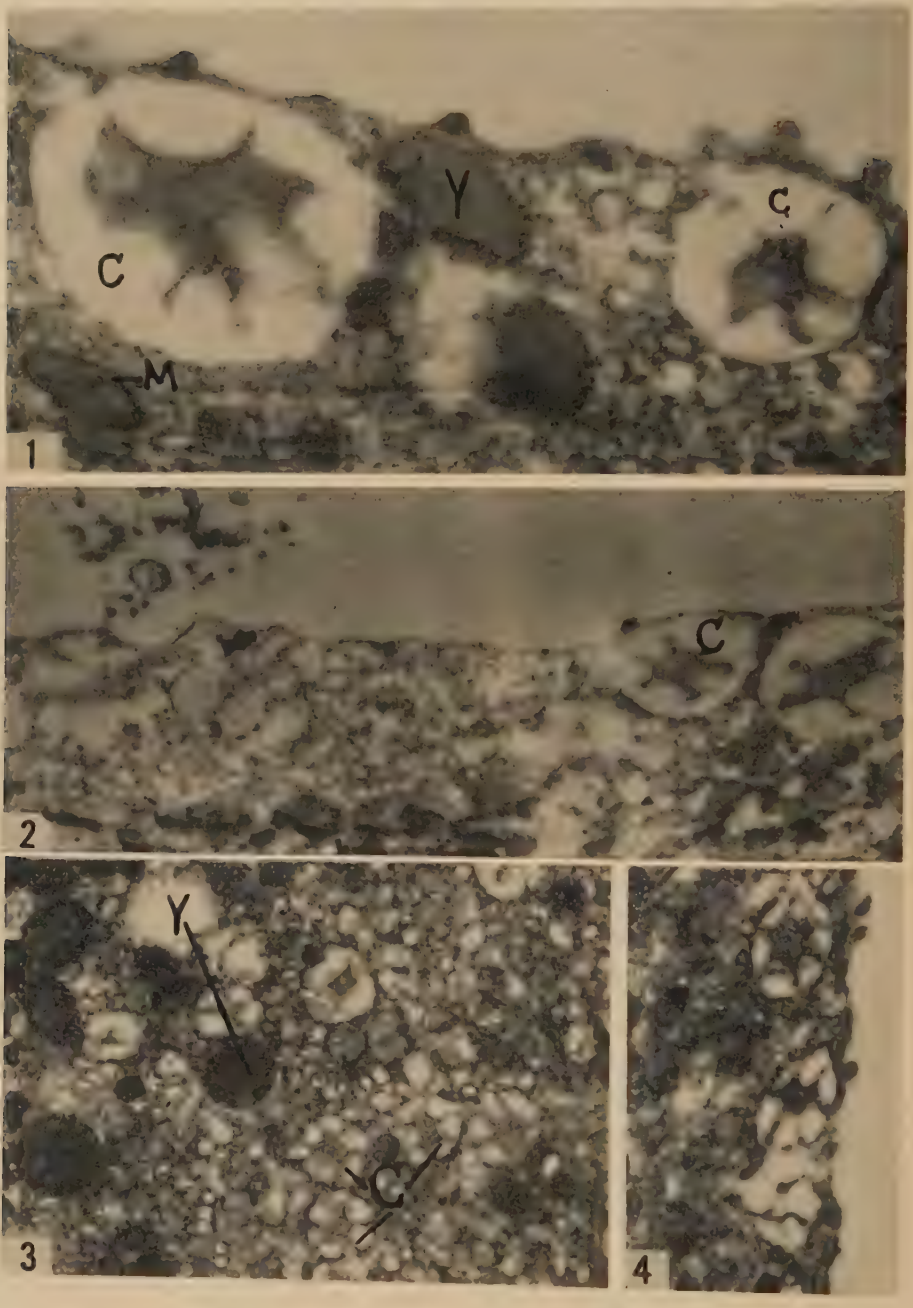
In a series of general and comparative studies by Cheney (4, 5, 7, 8, 9) of the effects of methylated xanthines upon the normal physiological processes and physical changes associated with the fertilization and growth of the *Arbacia punctulata* egg, it has been established that caffeine (1, 3, 7 trimethyl 2, 6 dioxypurine) is the most inhibitory in preventing fertilization. A M/10 caffeine-in-sea-water (CSW) concentration completely inhibits fertilization and growth.

In 1952, McCullough (19, 20) reported his electron microscopical observations on the *Arbacia* egg. Shortly afterwards, the fine structure of some unfertilized marine eggs (*Arbacia, Asterias, Spisula* (*Mactra*)) was studied by electron microscopy and reported by Lansing, Hillier and Rosenthal (16, 17). See also Hillier and Lansing (15). Based upon direct observations and photomicrographs via ordinary light microscopy, Cheney (9) suggested a parallelism between the degree of change in the cortical granules in the egg and the distribution pattern of certain intracellular granules, with the fertilizability of the egg. This current paper presents evidence via electron micrographs for the ultramicroscopic structural changes induced by caffeine in molarities known to retard and to inhibit completely the formation of the fertilization membrane (FM).

MATERIALS AND METHODS

All eggs utilized in each experiment were obtained from a single female (*A. punctulata*) whose sample egg volume showed 95 per cent fertilization. Eggs were shed and distributed in approximately equal numbers into 4 stender dishes containing 40 ml of solution. No. 1 was sea water (SW); No. 2, M/5,000 Caffeine-in-sea-water; No. 3, M/100 CSW; No. 4, M/10 CSW. After a 30 minute immersion of these mature,

[1] The experimental work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts.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unfertilized eggs in the experimental solutions at the temperature of running sea water in which the stender dishes were placed, the eggs were fixed for 1 hour in 1 per-cent osmic acid (O_8O_4) made up in sea water and buffered to pH_8 with Na-acetate-Na-veronal. The general procedures for fixation, dehydration, plastic embedding (methacrylate polymerization), cutting of thin sections (0.1 μ to 0.25 μ) by a microtome adapted for the use of glass knives (10), mounting on electron screens (150 copper electro mesh), and microscopy with an RCA (Model EMU) electron microscope with a 30 micra aperture in the objective lens, were according to Palade (23). Certain modifications which had been found by Hillier and Lansing (15) to be desirable for *Arbacia* eggs, were followed meticulously. Among the primary variations within the outline of Palade's procedures were the quick fixation (1 hr.), double washing, rapid dehydration (15-minute periods), in the use of a mixture (unpublished) recommended by Delbert E. Philpott, of two polymers (3 parts *n*-butyl to 1 part *n*-methyl methacrylate) as a plastic embedding material; and, in agreement with the experience of Hillier and Gettner (14) the plastic was not removed from the mounted specimens on the screens because such a procedure may produce drying artefacts of the type discussed by Anderson (1). The electron micrographs were made at original electronic magnifications of 3, 5, 7, and 9,000 × and enlarged photographically as indicated on the plates.

RESULTS

Physical characteristics of cytological morphology which showed consistently at electronic magnifications in both the control and experimental eggs were assumed to be typical structures unaffected by the chemical agent employed. Among such characteristics were the total mass proportions of groundwork, the shape and numbers of pigment and yolk granules and mitochondria, intra-groundwork "sheets" of a double membrane nature, etc. No significant change seemed to be apparent in these items. The finer structure, however, of all of these gross constituents of the cell was altered. The papillated surface of the egg was decreased progressively with increasing concentrations of caffeine until a smooth, non-papillated border was noted in M/10 CSW. Of the 3 experimental molarities employed, the M/5,000 CSW

Fig. 1. Normal *Arbacia* egg: Mature, unfertilized specimen. Section through periphery and showing the normal structure of the mitochondria, yolk granules, cortical granules and membrane papillae. Magnification 35,600 ×.

Fig. 2. M/10 Caffeine-treated egg: Section through periphery showing an exploded and two modified cortical granules. Note the disappearance of the surface membrane papillae. Magnification 17,800 ×.

Fig. 3. M/10 Caffeine-treated egg: Section through egg interior showing presence of a dense cluster of cortical granules and yolk granules at a distance below the surface. Magnification 16,500 ×.

Fig. 4. M/100 Caffeine-treated egg: Section near surface of egg showing a partial reduction of papillae per unit of the surface area. Magnification 15,700 ×.

Abbreviations: *C*—cortical granule; *M*—mitochondrion; *Y*—yolk granule.

showed no decrease in the papilla like nature of the egg surface and such eggs are fertilizable in equivalent percentage with the controls. In M/100 CSW, the surfaces were less papillate and fertilization of such eggs is retarded and development is abnormal (4, 5); and, in M/10 CSW, the eggs possess a smooth surface and such eggs are completely non-fertilizable.

The finely granulated configuration of the normal cortical granule matrix suggesting the "Hub" and "Spokes" of a wheel, appeared in caffeine-treated eggs to be in one of three variations of the original form: 1) irregular, rolled-paper appearance of the "Hub" area, 2) finely vacuolated "Hub", or 3) completely disintegrated state of the matrix. The mass groundwork of the egg likewise showed a very compact, minutely-alveolated structure in comparison with the controls. Echinochrome granules became swollen and transformed from an optical opaqueness to an appreciable degree of transparency. Mitochondria developed no apparent alteration and possessed, in many instances in both controls and the caffeine-treated, evidence of the double-membraned "sheet" phenomenon seen by previous investigators (2, 17, 24, 25, 30) in a variety of cells.

An increasing degree of clumping of yolk and pigment granules as reported earlier in light microscope studies by Cheney (4, 5) in the localization of yolk and pigment with progressively increased concentrations of caffeine was confirmed again in the electron micrographs. The *increasing* development of the degree of these morphological changes paralleled the *decreasing* fertilizability of the egg to complete non-fertilizability of the non-papillate eggs in the M/10 caffeine solution.

DISCUSSION

It is *obvious* from the *direct correlation*, between the degree of physical transformations in a number of different areas of the cell and the inhibition of the physiological processes associated with fertilization, *with* the concentration of the chemical agent, caffeine, employed, *that* several physico-chemical characteristics of the normal unfertilized egg and the finer structural sequences of its development have been modified. Observed changes are not due to the pH factor in view of the fact that the *Arbacia* egg was shown by Smith and Clowes (29) to be fertilizable and normal development occurred within a range of pH 6.0 to 8.3; and, Cheney (5) demonstrated that caffeine, even in its higher concentrations, does not increase the alkalinity of sea water more than 0.06 of a pH unit. In addition, in the preparation of both the control and experimental eggs in these current experiments, the fixative was

made up in sea water and the buffering required to adjust to pH_8 was accomplished by a Na-acetate-Na-vernal buffer. Negative results were obtained in so far as any influence of caffeine is concerned upon the scattered "sheets" and mitochondria. No alteration in the finer structure of mitochondria nor in the beaded, sheet partition-like structures in the groundwork and sometimes intra-mitochondrially was noted. Their appearance in *Arbacia* controls and experimentals corresponded with their description in a variety of normal cell types by Palade (24) in rat liver and other cells, by Porter, Claude, and Fullam (25) in tissue cultured cells, by Sjöstrand (30) in mouse kidney tubules, by Bernhard *et al.* (2) in an excellent treatise on cytoplasmic structures in general via electron microscopy, and by Weiss and Lansing (31) in the pituitary cells of the albino mouse.

The localization (clumping) of the pigment granules reported by Cheney (4, 5, 7, 9) as appearing within a shorter time with greater concentrations of caffeine has been noted again. In the current studies, even though the echinochrome pigment is dissolved and diffused out during fixation, an increased permeability of the membrane of the pigment granule is demonstrated by the fact that the electron micrographs show not only a double-membraned nature of the pigment membrane but also an increased size of these granules in the experimentals. In this connection it is of interest that, as the eggs are being fixed, there is optically a very clearly observable increasing gradient in the velocity of diffusion of the echinochrome from the eggs pretreated with higher caffeine molarities. For the greater compactness and more finely vacuolated appearance of the cytoplasmic groundwork, the ultimate gelation may be a partial explanation.

Reference was made by E. N. Harvey (12) to particles in the cortex of the *Arbacia* egg which were not moved by simple centrifugation. Later, Harvey (13) measured the tension necessary at the surface of the *Arbacia* egg to cause a 25 per cent increase in area and concluded that it is less than 0.2 dyne per cm with considerable variation in eggs. Runnström (28) associated the caffeine effect on the eggs of the sea urchin, *Echinocardium*, with surface activity and protein dehydration resulting in a viscosity decrease. Later he (28a) described an interesting conception of the role of cortical granules and their merging with the vitelline membrane as a factor in the formation of the fertilization membrane. Dan (11) described movement in the cortical layer as judged by the shift in pigment granules. Observations of the breakdown of cortical granules in an average of 8.5 seconds in a wave-like fashion from the point of stimulation to completion around the egg and the subsequent elevation of the fertilization membrane (FM) were

described by Moser (21, 22) in the *Arbacia* egg. More recently, Rothschild and Swann (27) observed via dark field cinematography that the cortex got brighter with fertilization whereas Moser reported reduced brightness. Simultaneously, Rothschild (26) reported that, accompanying surface contact by sperm, a change in diffusion phenomena occurred in both the the intracellular and cortical area of the egg. Evidence by Cheney (8, 9) via ordinary light microscopy suggests that caffeine initiates a change in the mass appearance of cortical granules. Electron microscopy in our current study substantiates this idea and reveals a degree of disorganization with greater concentrations which parallels the extent of retardation in the time factor of FM formation; and, drastic or complete disintegration of the cortical granule matrix occurs in M/10 CSW which also inhibits completely the FM formation in experimental cultures (5).

The mostly readily seen modification of the finer structure of the caffeine-treated egg is the disappearance of the numerous, papilla-like projections of the cytoplasm which constitutes the structural pattern of the egg surface as shown in detail by Hillier and Lansing (15). Such a physical change must involve surface tension phenomena and viscosity since the papillae, via electron microscopy, show direct continuity with the cortical cytoplasm. Although a viscosity study in 1949 by one of us (6), using the centrifugation method for the stratification of pigment granules and deformation of the *Arbacia* egg as criteria, reported no appreciable effect within a 30-minute pretreatment period by caffeine upon the viscosity of the internal protoplasm, the results did indicate some change in the total forces at the cell surface.

Brown (3) and Marsland (18) have used the centrifuge method as a means of determining cortical viscosity by the variation in the width of the pigment zone at the centrifugal end of the cell. This criterion assumes that there are red granules in the cortex of the unfertilized *Arbacia* egg until centrifuged. Wilson (32) challenges this assumption. After studying electron micrographs and observing pigment granules in the "peripheral area" of the *Arbacia* egg, it seems to the authors of this paper that one factor which is in need of clarification is an acceptable, exact definition of the limitation (depth), as well as the gel-type and gelation-state of the cortex. In any case, it now appears questionable whether this procedure utilizing this particular criterion for this purpose is sensitive enough to detect the quantitatively very small changes which may still be critical transformations in the viscosity capable of affecting the physiological behavior of the colloidal cortex. Wilson and Heilbrunn (33), in studying the protoplasmic cortex in relation to stimulation,

suggest that all cells have a cortex differentiated from the internal protoplasm and that the course of development of a marine egg may depend on the physical arrangement of the cortex. Logically, in our experiments, the outer border and cortex would be the first to become affected by the chemical agent added to the environment. Their evidence is based primarily upon the *Chaetopterus* egg in which the cortical details are determined more readily than in the *Arbacia* egg because of the much smaller size of the cortical granules in the latter. However, they report that the behaviour of the *Arbacia* cortex is similar to the *Chaetopterus* egg changes in viscosity in that all agents which stimulate the egg to undergo development cause a *liquefaction* of the cortex followed by a series of increases and decreases in viscosity with time as development proceeds. Whatever the complete explanation may be of the inhibitory effect of caffeine upon fertilization and development, one factor would seem to be its interference with the degree and sequence of these alternating rigidities of the cortical protoplasm. The total volume of the caffeinized egg does not vary significantly within the time-treated period employed (5). These observations, especially any liquefaction, could account theoretically for the smoothing out of the cell border by the existing surface tension or, but not required necessarily, by an increase in the total surface forces.

SUMMARY

This study of the effect of caffeine upon the physiological processes underlying fertilization in the *Arbacia* egg by means of the physical changes in the finer structure of the cell, as revealed by electron microscopy, indicates the following.

Negative results were obtained with reference to mitochondria and to the beaded, sheet-partition-like, double membranes scattered through the cytoplasm. These structures have been called sheets, fibers, endoplasmic reticulum, etc. These synonomous terms of different authors have been shown by Bernhard *et al.* (2) to be basophilic substances. No evidence was obtained that caffeine affects them.

Positive results were observed showing caffeine effects as follows:

1. The most drastic alteration of the ultramicroscopic structure of the mature, unfertilized *Arbacia* egg as shown by the electron micrographs of caffeine-treated eggs, is the disappearance of the papillated surface border.

2. The degree of modification of the finer structural details is directly proportional to the concentration of the caffeine employed.

3. The groundwork matrix of the egg becomes more compact and more minutely vacuolated.

4. Pigment granules possess a double membrane which becomes more permeable to the release of echinochrome molecules; and the granule itself becomes swollen.

5. The cortical granule matrix, especially the "Hub" area of the wheel-like "Hub" and "Spokes" configuration of the normal cell, becomes disorganized into one of three variations of its original form.

6. An explanation is suggested for the change in the papilla-like border.

The authors wish to express their appreciation to Delbert E. Philpott for his cooperation and preparation of the electron micrographs.

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Experimental Cell Research, **8**, *181–187 (1955)*

MITOSIS IN CULTURES OF NEWT TISSUES

IV. THE CELL SURFACE IN LATE ANAPHASE AND THE MOVEMENTS OF RIBONUCLEOPROTEIN

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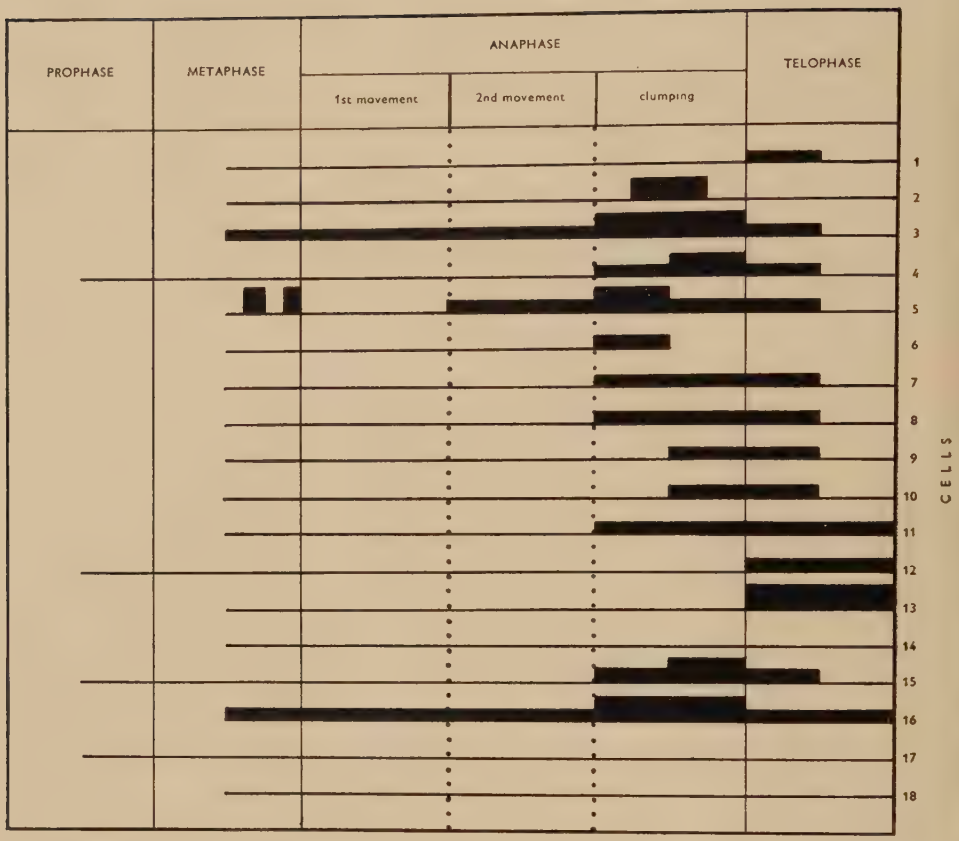
Cells in tissue culture often bear small, transient cytoplasmic protrusions during mitosis. Such protuberances are clearly seen in the chick fibroblast depicted by Strangeways (12), and, as pointed out by Hughes (7), are usually evident in cinematograph records of mitosis. The present studies on this "bubbling" have been made on living fibroblasts in cultures of newt tissues and it has been possible to distinguish sufficient detail in this material to observe cytoplasmic and nuclear events simultaneously. Thus the time relations between structural changes at the cell surface and those in the chromosomes could be recorded. These observations on living cells were combined with studies on fixed preparations stained to show ribonucleoprotein (RNP), and correlation was found between the surface changes and alterations in the distribution of the RNP during mitosis.

METHODS

The methods of tissue culture and observation were the same as those previously described (1). Whole cultures were fixed in methanol and stained with a combination of May-Grünwald and Giemsa stains, which gives a differential and specific colouration for ribonucleoprotein (RNP) and desoxyribonucleoprotein (9). Cultures were also stained with a mixture of methyl green and pyronine at pH 4.5. Digestion with ribonuclease, kindly supplied by Dr. M. Webb, was used to confirm the presence of RNP in different regions of the cells, cf. (9).

OBSERVATIONS

During mitosis, especially in late anaphase, the surface of the newt fibroblasts bore small rounded processes, 1–5 μ in diameter, which were protruded and retracted asynchronously. These cytoplasmic "bubbles" occurred on the cell body and rarely, if ever, exceeded one-fifth of the whole cell in dia-



Text-fig. 1. Times at which "bubbles" occur at the surface of the cell body. Based on 18 unselected series of photographs originally taken for other purposes. Each horizontal line represents the period over which a cell was observed. The divisions of anaphase correspond to the two chromosome movements described in the third paper of this series and to the period of apparent clumping of the chromosomes just before telophase.

meter; an individual knob was usually completely retracted within ten minutes of its first appearance. Newt fibroblasts in mitosis formed two other types of cytoplasmic protuberance from which those described in this paper must be distinguished. Firstly, as already recorded (2), after the cell had rounded up in metaphase, any unretracted large processes became narrower and acquired many small knobs. These knobs did not continually form and disappear, but remained unchanged in appearance until the outline of the process became more regular during the re-expansion of the cell. Secondly, in a cell which had been damaged, e.g. by over-illumination, so

that the metaphase chromosomes clumped together, sluggishly moving, elongated cytoplasmic processes were formed but not withdrawn; this very uncommon change in the cytoplasm differed greatly from the rapidly changing, unstable knobs which formed on the cell body during the "bubbling" of normal mitosis.

This "bubbling" of the cell surface during mitosis is not peculiar to tissues cultivated *in vitro*. In sections of a 4-day chick embryo, similar protrusions were found on cells in anaphase in the neural tube and in the mesenchyme. "Bubbling" was also observed during the mitosis of a mesenchyme cell in the tail of an intact living newt tadpole; this was the only mesenchyme cell seen in mitosis in a living tadpole, although epithelial cells in division were often encountered.

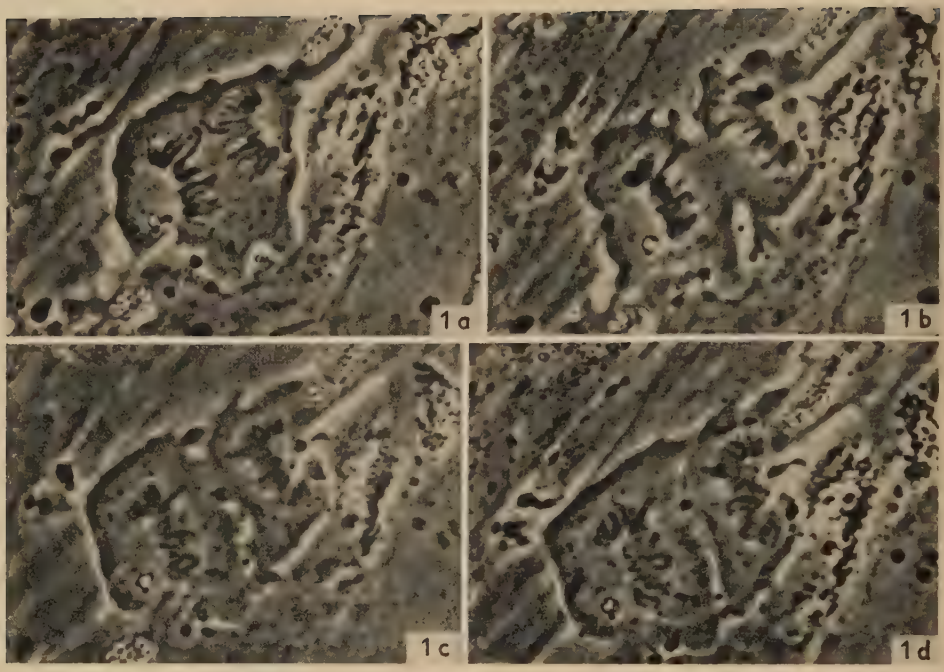
It is evident from Text-Fig. 1 that in the newt tissue cultures "bubbling" occurred mainly at the end of anaphase, when the daughter chromosomes appeared to be clumped together within their respective groups, and during early telophase. The disturbance of the cell surface was greatest at the end of anaphase and steadily decreased during telophase (Fig. 1).

Three out of the eighteen cells (Nos. 3, 5 and 16), from photographs of which Text-Fig. 1 was derived, "bubbled" in metaphase; the protuberances, however, were found almost entirely in the zone of cytoplasm overlying the edge of the metaphase plate, i.e. where the cell surface was nearest to the chromosomes. During anaphase or telophase, whenever the surface was more disturbed in one part than in another, the greatest disturbance was nearest to the underlying chromosomes (Fig. 2).

The distribution of RNP in newt fibroblasts in culture underwent the following changes during mitosis. During prophase each chromosome acquired a charge of RNP, which formed a matrix in which the two chromatids were embedded. This RNP may have been derived, at least in part, from the fine granules ($< 0.5\ \mu$ in diameter) which were scattered through the nucleoplasm in interphase. RNP often appeared first on parts of the chromosomes at some distance from the nucleoli.

In living cells, the nucleoli, which were rich in RNP, became eroded and dwindled until during the last half-hour of prophase they were no longer detectable. Stained preparations showed that the chromosomes had frequently acquired some RNP before changes in the shape of the nucleoli were evident. With the final disappearance of the nucleoli, the nucleocytoplasmic boundary ceased to be distinguishable in either living or fixed cells.

In preparations stained for RNP, the concentration of the latter at anaphase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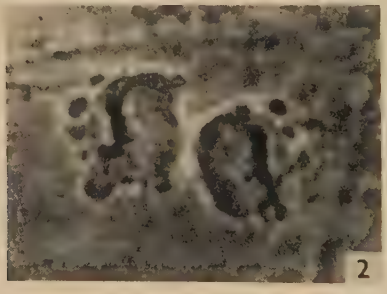
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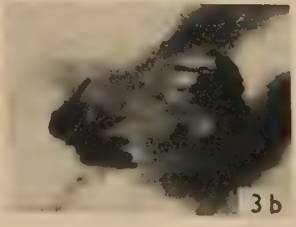
3b

was sometimes seen to be highest in the equatorial region between the separating groups of daughter chromosomes from which it appeared to have been shed (Fig. 3). In many cells staining failed to show the equatorial RNP, probably for two reasons. In the first place the general cytoplasmic RNP sometimes caused such an intense colouration that a moderate local increase would be masked, while in other cells the beginning of cleavage made the equatorial region so shallow that any increase in the concentration of stain would be compensated by the reduction in the total thickness of cytoplasm. In general, where neither of these factors applied, the staining indicated an increased concentration of RNP in the equatorial region during anaphase.

Examination of cells in late anaphase and early telophase showed that the cytoplasmic protuberances already described arose at sites where the concentration of RNP was high (Fig. 3). In young daughter nuclei formed during early telophase the RNP lay in the interstices between the chromosomes. After the formation of the nuclear membrane, "bubbling" rapidly subsided.

DISCUSSION

The findings summarised in Text-Fig. 1 indicate a tendency for transient irregularities of the cell surface or "bubbling" to occur during the last part of anaphase and early telophase, while examination of an individual cell usually showed that the "bubbling" steadily decreased after each group of daughter chromosomes had become enclosed by a nuclear membrane. This synchronisation between the formation of a nuclear membrane and the diminution of bubbling suggests that "bubbling" may be evoked by the arrival at the cell surface of a substance released from the clumped chromo-

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Fig. 1. Living cardiac fibroblast. Phase contrast. × 750. (*a*) Anaphase. (*b*) The chromosomes are clumping together and small rounded projections have formed at the surface of the cell. (*c*) The nuclear membrane now encloses the chromosomes; some small "bubbles" are still present on the opposed surfaces of the daughter cells. (*d*) The chromosomes become thinner; there is no further "bubbling" at the cell surface.

Time intervals: (*a*)–(*b*) = 19 minutes; (*b*)–(*c*) = 5 minutes; (*c*)–(*d*) = 6 minutes.

Fig. 2. Cardiac fibroblast. The clumped chromosomes of late anaphase lie eccentrically, and the cell surface shows "bubbling" mainly where it most closely overlies the chromosomes. Phase contrast. × 750.

Fig. 3. Cardiac fibroblast. (*a*) Living cell in anaphase. There are rounded protrusions of the cell surface at the lower right-hand corner of the photograph. Phase contrast. × 800. (*b*) The same cell, at the same magnification, fixed in methanol within half-a-minute of (*a*), and stained with May-Grünwald and Giemsa's solutions. The blue colour of the cytoplasm due to RNP is intense between the chromosomes, and in the region where "bubbling" was seen.

somes and able to enter the cytoplasm only while the chromosomes remain unenclosed by a nuclear membrane.

An association between the chromosomes and the disturbances at the cell surface is shown by the three cells which, as indicated in Text-Fig. 1, "bubbled" in metaphase; the part of the surface chiefly affected was that nearest to the chromosomes, i.e. it was the zone overlying the edge of the metaphase plate. Similarly, in anaphase any eccentricity in the position of the chromosome groups within the incipient daughter cells corresponded to an eccentricity in the distribution of "bubbling" at the cell surface (Fig. 2). Another example of the deformation of the cell surface in the neighbourhood of chromosomes is to be found in the work of Hughes and Fell (8), who found that during a mitotic division disturbed by mustard gas, a chromosome dissociated from the others and lying near the cell surface was accompanied by strictly local cytoplasmic "bubbling".

The observations made on the distribution of RNP during mitosis confirm the findings of other workers and also throw some light on the possible nature of the substance which, when released from the chromosomes, appears to excite the formation of transient cytoplasmic protuberances. Frolova (6), Ris (11), and Kaufmann, McDonald and Gay (10) have demonstrated ribonucleic acid as a component of metaphase chromosomes in plant and invertebrate cells. Jacobson and Webb (9) found that in cells of the chick, mouse and man, the chromosomes in prophase acquire a charge of RNP which they shed in anaphase. Davies (4) showed that in wing fibroblasts in culture, the equatorial region of the cytoplasm through which the chromosomes had moved during anaphase, showed ultra-violet light strongly. In the present studies it has been further demonstrated that there may be a concentration of RNP in the neighbourhood of "bubbles" and also between the chromosomes in telophase. It therefore seems possible that RNP may be the material which is shed from the chromosomes in anaphase, activates "bubbling" at the cell surface and is trapped within the daughter nucleus by the formation of the nuclear membrane.

It is interesting that RNP should be concentrated in the equatorial region of the cell at the same time that two structural changes, *viz.* cleavage and elongation of the interzonal part of the spindle, are taking place in that region, cf. (2). Swann (13), on the basis of alterations in birefringence in sea urchin eggs during cleavage, has suggested that certain structural changes in the cytoplasm may arise in response to the presence of active substances released from the chromosomes. Dustin (5) has argued from observations on vacuole-formation, that RNP is associated with changes in cell structure.

Dalcq, Pasteels and Fautrey (3) have found high concentrations of ribonucleoprotein in locomotor protrusions and other surface irregularities of embryonic cells.

Text-Fig. 1 includes three cells (Nos. 14, 17 and 18) which showed no "bubbling" in mitosis. All were perichondrial fibroblasts, but cells of this type usually "bubble" in late anaphase, and it was not possible to correlate the absence of protrusions with any other features of the three cells.

SUMMARY

1. Fibroblasts in cultures of newt tissues have been observed alive and after fixation and staining with either May-Grünwald and Giemsa stains, or a mixture of methyl green and pyronine at pH 4.5.

2. In late anaphase and in telophase transient protuberances or "bubbling" appear at the surface of the cell; these are not peculiar to tissue cultures.

3. The protuberances are related temporally to nuclear changes and spatially to the chromosomes in ways that suggest that they arise in response to an agent released by the chromosomes.

4. The staining of the fibroblasts suggests the possibility that a ribonucleoprotein may be the agent responsible for "bubbling".

I am most grateful to Dr. H. B. Fell, F.R.S., for help and instruction during all these studies. I also wish to thank Dr. J. Chayen of King's College, London, for his friendly advice on the interpretation of stains used to locate nucleic acids. This work was done during the tenure of a research studentship from the Medical Research Council.

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Experimental Cell Research, **8**, *188–212 (1955)*

GRADUAL TRANSFORMATION OF SOLID INTO ASCITES TUMORS. EVIDENCE FAVORING THE MUTATION-SELECTION THEORY[1]

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In previous experiments (14, 16, 20, 21) concerning the gradual transformation of certain solid tumors into ascites form (A-form)[2] some indirect evidence was obtained indicating that the change was probably due to the selective overgrowth of a small number of cells, better suited for multiplication in the peritoneal fluid than the majority of the original tumor-cell population and not to a physiological adaptation process induced by the environment in the population as a whole. It was demonstrated that the change is stable and is maintained even during prolonged propagation in the original solid form.

For convenience of reference, the two main alternative possibilities of transformation will be referred to as the mutation-selection versus the adaptation hypothesis. The term mutation is being used in its broadest sense, meaning stable heritable cellular changes without regard to their intra- or extrachromosomal nature.

The present paper describes further experiments and presents more direct evidence favoring the mutation-selection hypothesis. The permanence of the transformation is demonstrated in a more quantitative way, with the help of growth curves. In addition, some comparisons of various characteristics of the original and changed cell populations are given.

MATERIALS AND METHODS

Mice.—The animals used for the present experiments belonged either to the C3H/St strain or were F_1 hybrids produced by mating animals of the C3H/St strain with mice from one of the following three inbred strains: A/St, DBA/2 and C57BL.

[1] The publication of this paper was made possible by the aid of local grants.

[2] *Abbreviations.* -A-form: growth in ascites form, defined as the *multiplication* of free tumor cells and/or tumor-cell complexes in the peritoneal fluid, leading to the accumulation of ascites containing a high percentage of tumor cells in nearly pure culture. DNA: desoxypentosenucleic acid. KRPG: Krebs-Ringer-phosphate, containing 0.25 per cent gelatin, pH – 7.4. For the abbreviations concerning the various tumor sublines, see Tumors.

All four inbred strains were received from Dr. T. S. Hauschka in 1950 and were kept since then by 12 (C3H), 9 (A/St), 8 (DBA) and 8 (C57BL) generations, resp., of brother-to-sister mating in our laboratory. All animals were kept on a standard checker diet which, as well as drinking water, was available *ad libitum* at all times. Animals of both sexes were used at the age of 2–3 months and with a body weight of 16–22 g.

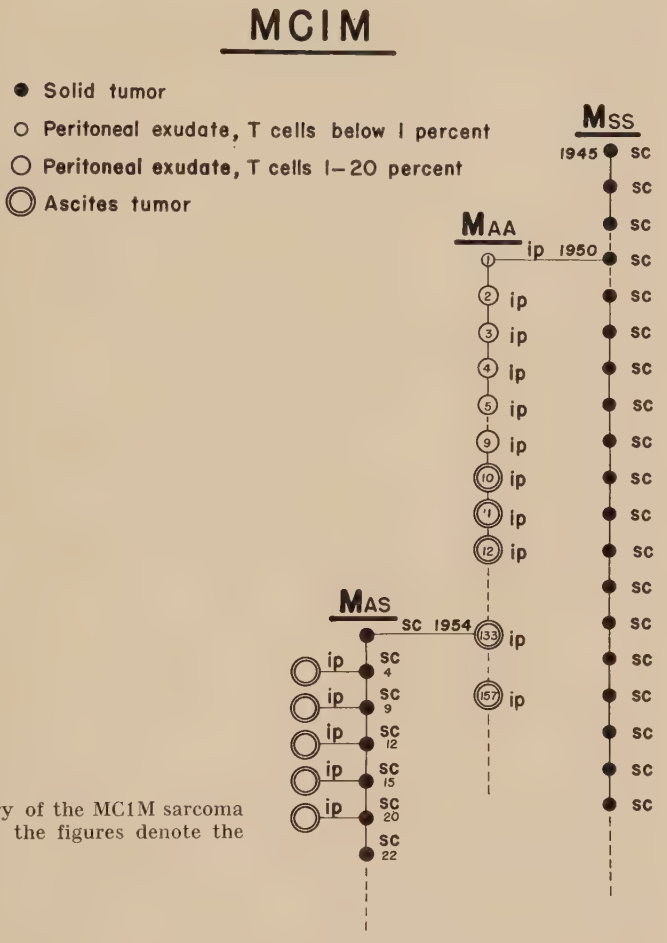


Fig. 1. Transplantation history of the MC1M sarcoma sublines. For M_{AA} and M_{AS}, the figures denote the number of serial passages.

Tumors.—The same two sarcomas (MC1M and MC1A) were used as in the previous paper (14). The original solid line of both tumors is kept by serial subcutaneous transfer and is designated M_{SS} and A_{SS}, resp. They can be converted into typical ascites tumors only gradually by serial intraperitoneal transplantation of peritoneal exudate (18). For MC1M, about 10 "fluid transfer generations" (18) were necessary before abundant free-cell growth was obtained in the peritoneal fluid, while MC1A needed

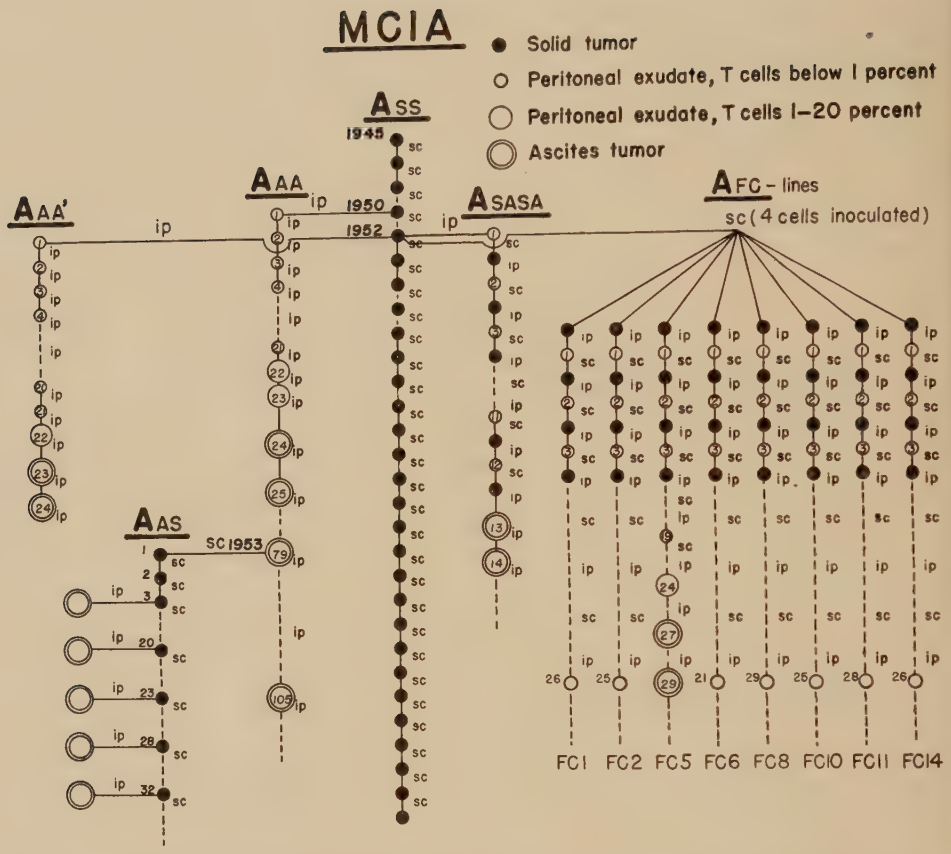


Fig. 2. Transplantation history of the MC1A sarcoma sublines. The small figures denote the number of serial transfers.

24 similar transfers. The ascites lines of both tumors are kept by serial i.p. transfer of ascitic fluid and are called M_{AA} and A_{AA}, resp. Furthermore, a solid transfer line has been established from both ascites lines by subcutaneous inoculation of ascitic fluid and subsequent serial transfer of the solid tumor. These lines are called M_{AS} and A_{AS}. The more detailed history of the various lines appears in Figs. 1 and 2 (MC1M and MC1A).

The ascites form of MC1M (M_{AA}) contains exclusively single cells. During the majority of the growth period between 80 and 90 per cent of the population consists of free tumor cells. These are easily distinguishable due to their large size, high nucleo-cytoplasmic ratio and huge nucleoli (Figs. 3–5). Infiltration of the solid intra-abdominal tissues is rather inconspicuous until the latest stages of the growth period. Growth curves describing the multiplication of free tumor cells have been published for this tumor (22).

The ascites line (A_{AA}) of MC1A regularly contains a number of small cell clumps in addition to free cells. Smaller amounts of ascites are obtained with this tumor than with M_{AA}, solid infiltration is more pronounced and survival time somewhat longer. Cell morphology is rather similar to M_{AA} (Figs. 6–7).

Methods of inoculation.—Routine transfer of solid tumor lines was performed by subcutaneous inoculation of fine tumor mince, suspended in KRPG. The ascites lines were maintained by serial intraperitoneal transfer of 0.2–0.4 ml ascitic fluid.

Chemical methods.—DNA was determined by the method of Schneider (31). For the calcium determinations, 5–10 grams of tumor tissue was dried to constant weight. The amount of dry substance was between 1 and 2 gram. This was incinerated at 400°C in an electric oven for six hours. The white ash was dissolved in dilute hydrochloric acid. Calcium was precipitated in the form of oxalate. The washed precipitate was dissolved in hydrochloric acid and oxalic acid was determined by titration with $KMnO_4$.

Quantitative determination of tumor-cell number in the peritoneal fluid.—The total number of tumor cells in the peritoneal fluid has been determined quantitatively at successive intervals after the inoculation of known numbers of cells. Different procedures of inoculation were used for solid and ascites tumors and the estimation of cell number had to be modified for lines of MC1A which all contain cell clumps in addition to free cells. The following procedures were used:

1. Inoculation of ascites cells: 2–5 mice showing marked abdominal distension were killed by cervical dislocation. Their ascites was collected with a syringe and pooled in a container immersed in an ice bath. One aliquot was diluted two hundred times with Tyrode's solution, containing 0.05 per cent eosin (32). Cells were counted under phase contrast in a Buerker hemocytometer. Tumor cells of MC1M and MC1A are easily identifiable because of their large cellular and nuclear size and huge nucleoli (Figs. 5 and 7). Eosin-stained and unstained cells were counted separately. The percentage of eosin-stained cells was usually of the order of 1–3 per cent and never exceeded 10 per cent. A predetermined number of eosin-unstained tumor cells was used for inoculating groups of 25–30 mice. Dilutions were made with KRPG and 0.2–0.3 ml was introduced into the peritoneal cavity with a tuberculin syringe.

For ascites lines of MC1A the procedure was complicated by the presence of small cell clumps in the fluid. These could be removed by filtration through a 100-mesh stainless screen. The resulting suspension contained mostly single cells and occasional groups of a few cells.

2. Inoculation of solid tumor cells: Cell suspensions were prepared by the method of Kaltenbach (12). The minced tumor tissue was forced through a 20-mesh stainless steel screen. The material was suspended in KRPG and passed through a 60-mesh-screen. The resuspended cells were filtered through a 100-mesh screen. The temperature was kept near 0°C during the whole procedure. One aliquot of the final suspension was used for cell counting as described above for the ascites cells. The proportion of eosin-unstained cells was usually of the order of 20–30 per cent. A predetermined number of eosin-unstained cells was used for inoculation.

3. Determination of the total number of free tumor cells in the MC1M sublines: The procedure used was essentially the same as that described by Klein and Révész (22). Animals were killed at successive intervals after inoculation and their peritoneal fluid was collected. The abdominal cavity was rinsed subsequently with saline and

the washing fluid was added to the same pool. Rinsing continued until a total volume of 30–40 ml was reached and the washing fluid became clear. The volume of the combined fluid was determined and one aliquot was used for the estimation of cell concentration in a Buerker hemocytometer. Another aliquot was centrifuged and the cellular sediment was examined on Papanicolaou smears. By differentially counting tumorous and non-tumorous cells, their total number in the peritoneal fluid could be calculated.

Another aliquot of the combined fluid was used for DNA determinations. It was divided into two known volumes (usually about 10 ml each) and centrifuged. The supernatant was discarded and 5 ml of a 10 per cent trichloroacetic acid solution was added to the sediment. Temperature was kept near 0°C during the whole procedure. Parallel determinations of DNA were made on the two samples by the Schneider technique (31).

4. Estimation of tumor cell number in the peritoneal fluid for lines of MC1A: As MC1A contains cell aggregates besides free tumor cells, a direct counting procedure could not be used. The peritoneal cavity of the mice was rinsed with saline, and the DNA content was determined on two aliquots as described above. A third aliquot was centrifuged and a smear preparation of the sediment served for differential counting in the same way as for MC1M. The estimation of cell number from the data will be described in the next chapter.

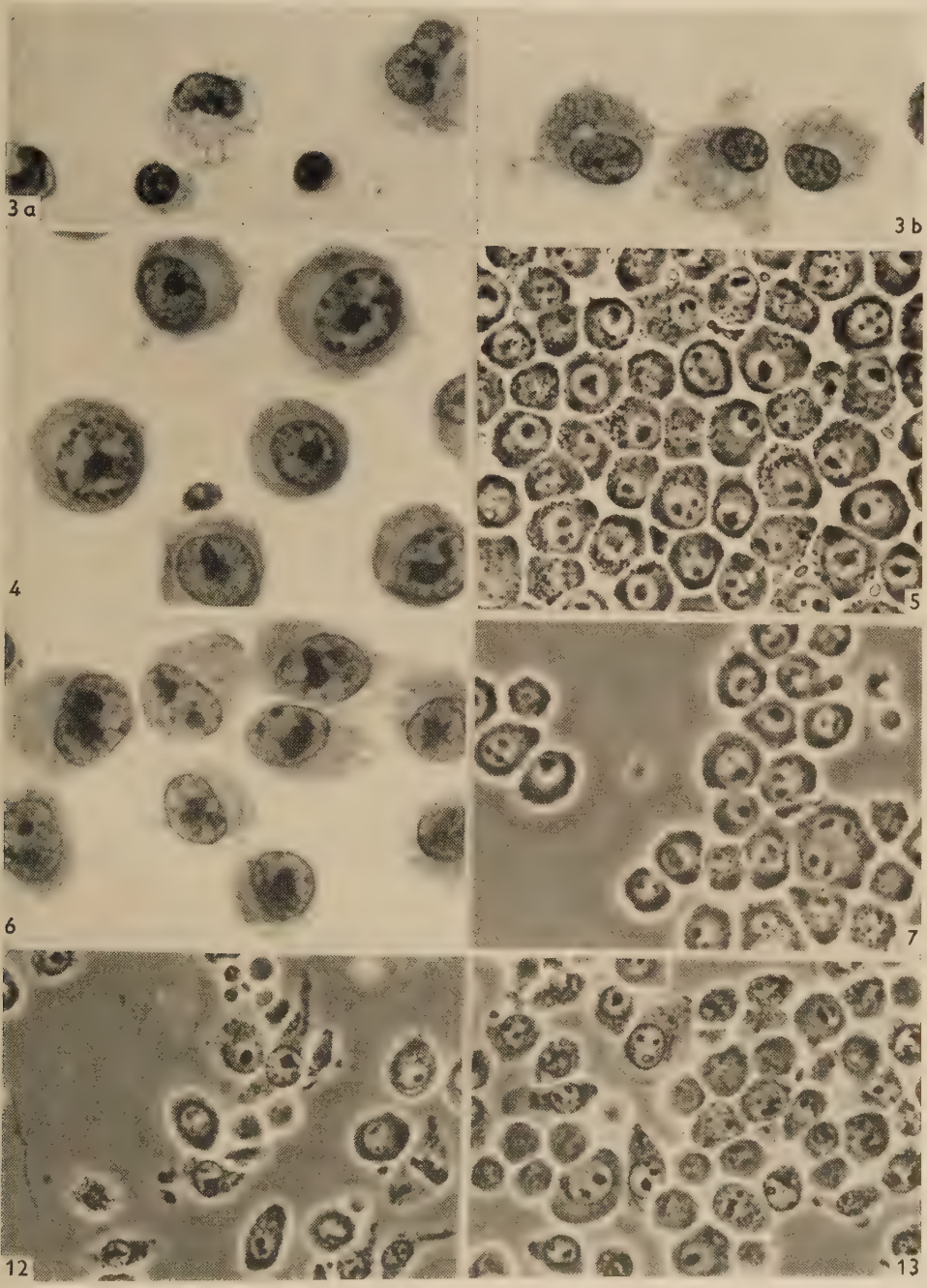
RESULTS

Estimation of tumor-cell number from DNA determinations

a) *Error of the DNA estimation.*—This was established from the differences of 64 duplicates. The error of a single estimation (σ_s) was found to be 7.8 per cent while the mean of two parallel determinations had a standard error (σ_M) of 5.5 per cent.

b) *Determinations on MC1M sublines.*—The mixture of lymphocytes, leukocytes, histiocytes and mesothelial cells contained in the peritoneal fluid of normal mice represents a predominantly diploid cell population (18). A series of DNA determinations on such material by the method used in the present investigation gave the average value of 6.8×10^{-9} mg DNA per cell. This is in good agreement with the values of other authors for diploid mouse cells (4).

Fig. 3. *a* and *b*. Papanicolaou smear of the peritoneal fluid from normal mice, showing lymphocytes, mesothelial cells and histiocytes. Magnification: 1,200 ×.
Fig. 4. Papanicolaou smear of the MC1M ascites line (M_{AA}). Magnification: 1,200 ×.
Fig. 5. Same as Fig. 4 in the phase contrast microscope. A drop of fresh ascites was placed on a slide and slightly squashed with a cover slip. Magnification: 500 ×.
Fig. 6. Papanicolaou smear of the MC1A ascites line (A_{AA}). Magnification: 1,200 ×.
Fig. 7. Same as Fig. 6 under phase contrast. Magnification: 500 ×.
Fig. 12. Cell suspension prepared from a solid M_{SS} tumor under the phase contrast microscope. Magnification: 500 ×.
Fig. 13. Cell suspension made from M_{AS} solid tumor. Phase contrast. Magnification: 500 ×.


3 a
3 b
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MC1M is an aneuploid tumor with a modal chromosome number between 65 and 70 (26). Calculated from the data of Levan and Hauschka, the mean chromosome number per cell is about 87, provided that frequency distributions of metaphase chromosome numbers reflect the distribution of ploidy in the population. As a direct relationship seems to exist between chromosome number and DNA content (33), a population of MC1M cells can be expected to contain a mean amount of 14.8×10^{-9} mg DNA per cell. (The diploid chromosome number of the mouse is 40.) This is only an approximation, as the actual average DNA value will be influenced by the number of cells having doubled their DNA in preparation for mitosis and by possible shifts in the proportions of cells with different chromosome numbers during various periods of growth. The first factor is probably of no major quantitative significance for the average value of the population, however, as it has been shown that rapidly growing diploid tumors have nearly the same average DNA value per cell as resting diploid cells (19). Also, a good correlation has been found between the mean chromosome number and average DNA content of various ascites tumors (18, 8, 26). Furthermore, the DNA value calculated presently from chromosome numbers (14.8×10^{-9} mg) is in good agreement with direct determinations on this tumor (18), giving the average value of 14.0×10^{-9} mg per cell. With regard to possible shifts in the proportion of cells with different chromosome numbers, the maintenance of characteristic chromosome spectra through successive transfer generations, as demonstrated by the work of Hauschka and Levan, suggests that shifts within one generation would, if they occurred, probably be of comparatively small significance for the average of the population.

The number of tumorous and non-tumorous cells was estimated from the DNA values by the following formula:

$$\mathrm{DNA} = 14.8 \times 10^{-9} \times T + 6.8 \times 10^{-9} \times E$$

where DNA is the total amount of DNA in mg in the combined rinsing fluid, T is the total number of tumor cells and E is the total number of non-tumorous exudate cells. The percentage of tumor cells (t) and of exudate cells (e) was determined by differential counting. Obviously,

$$E = \frac{e}{t} \cdot T.$$

Hence,

$$T = \frac{\mathrm{DNA} \times 10^{9}}{6.8 \frac{e}{t} + 14.8}.$$

As the total number of tumor cells could also be determined directly by cell counting, the values estimated from DNA determinations could be checked against results obtained by a direct and independent method. Fig. 8 shows the multiplication of M_{AS} cells in the peritoneal fluid, measured

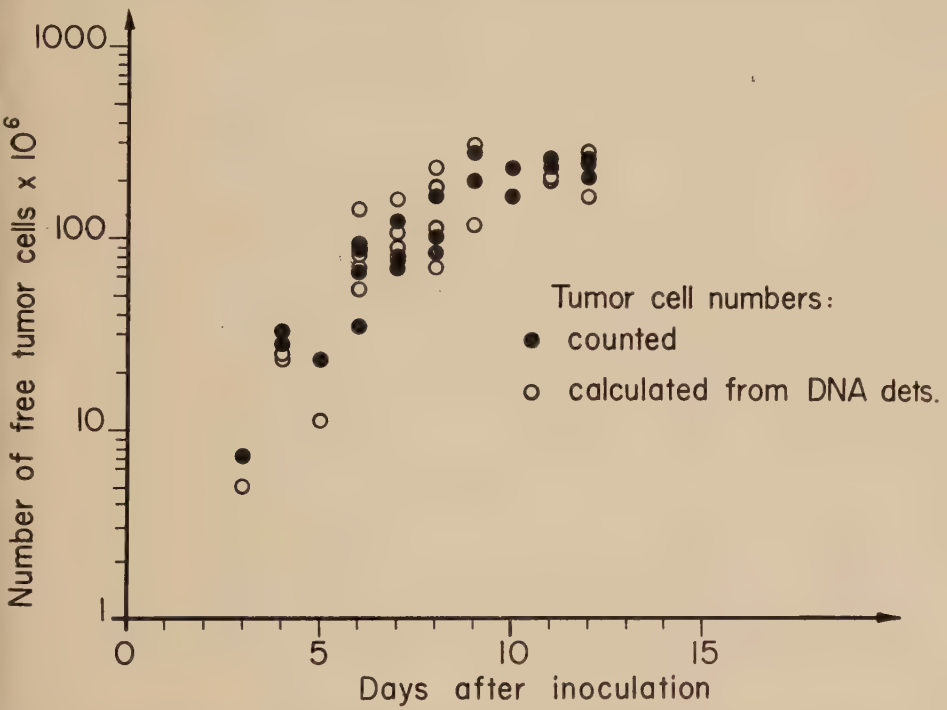

Fig. 8. Growth of free M_{AS} cells in the peritoneal fluid, as measured by cell counting and DNA determinations, respectively.

by both methods. Fig. 9 shows the correlation between the two methods ($n = 46$; $b \pm \sigma_b = 0.94 \pm 0.05$; $r = 0.94$; $z \pm \sigma_z = 1.74 \pm 0.15$).

In order to compare the error of the estimate for both methods the following calculations were carried out:

Sixteen parallel determinations were available on pairs of mice. Each pair was inoculated simultaneously with the same number of cells and sacrificed at the same time. The total number of free tumor cells was determined by both methods in all mice. The logarithms of tumor cell numbers were used for calculation. The mean of all 16 pairs was 8.149 (range 7.444–8.823) by cell counting and 8.131 (range 7.374–8.630) as calculated from DNA values. The variability involved in a single estimation (cf. (22)) was

determined from the differences between mouse pairs, and was found to be 0.1085 logarithmic units for cell counts and 0.1185 for DNA. The difference is not significant ($t = 0.34$). It has to be concluded that the random errors of both methods are of a similar magnitude.

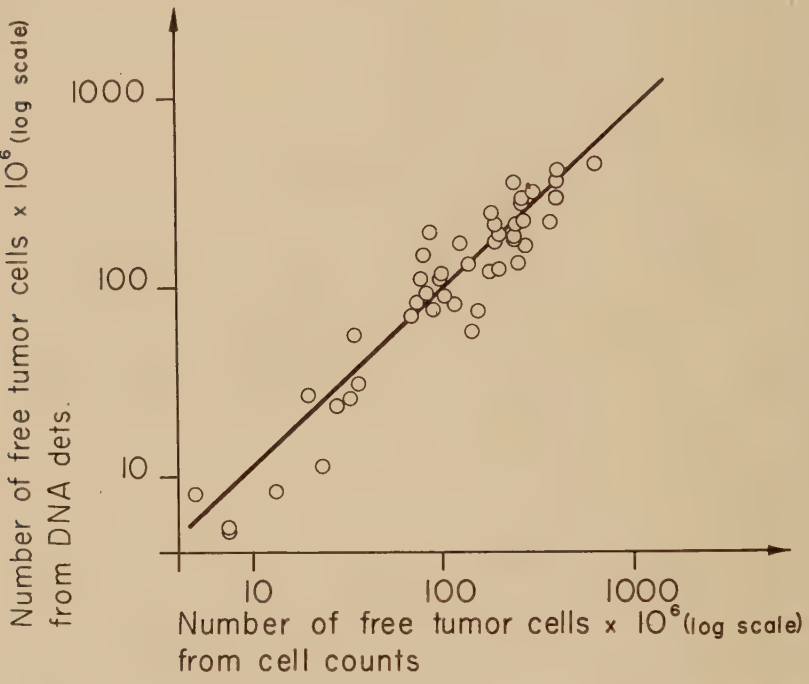

Fig. 9. Correlation between the total number of free tumor cells during the intraperitoneal growth of M_{AS} as calculated from DNA values and determined by cell counting, resp. The straight line has been fitted to the data by the method of least squares.

In order to detect possible systematic errors involved in the DNA method, a comparison was made between corresponding values, obtained by both methods on the same sample. The values calculated from cell counts were subtracted from those obtained by the DNA method. For 46 pairs of values, the mean difference was -0.025 ± 0.015 logarithmic units. Testing the deviation of the mean difference from zero, a t value of 1.70 is obtained, indicating a statistically probable ($0.1 > P > 0.05$) deviation. A slight systematic error is thus probably involved in the DNA determinations, tending to give somewhat lower cell numbers than direct counting. This may depend on a certain inaccuracy of the average DNA value used for calculation. For the purposes of the present work, a systematic error of this magnitude lacks significance.

c) Determinations on MC1A sublines.—For MC1A, DNA determinations could not be controlled by direct cell counts. From the experience gained with MC1M it was considered justified to use the DNA method as the only means to determine the total number of tumor cells. As the average chromosome number of MC1A is about 96 (26), the value of 16.3×10^{-9} mg was assumed to approximate the DNA content of the average tumor cell. Ultraviolet spectrophotometry of isolated nuclei (G. Klein, unpublished) gave an average value of $15.5 \pm 1.08 \times 10^{-9}$ mg DNA which agrees reasonably well with the calculated value.

Calculations were carried out in the same way as with MC1M.

Permanence of the ascites transformation

In a previous paper (14) experiments were reported indicating that conversion to A-form is of permanent nature, being retained even under the conditions of serial subcutaneous passage in the solid form. The findings were based on comparisons between M_{AS}, A_{AS} and their original solid lines, M_{SS} and A_{SS}, respectively. Both AS lines were carried through an additional number of serial solid transfers since the previous experiments (cf. Figs. 1 and 2), thereby increasing the possibility of reverse changes. The growth curve method permits, furthermore, a more quantitative comparison between the ascites lines and their AS sublines carried by serial solid transfer. For these reasons the following experiments were carried out:

a) Comparison of M_{AS_1}, $M_{AS_{15}}$, M_{AA} and M_{SS}. — The designation M_{AS_1} is applied to solid tumors derived directly from line M_{AA} by subcutaneous inoculation of ascitic fluid. $M_{AS_{15}}$ was originally started in the same way and was subsequently carried through 14 additional subcutaneous transfers in the solid form. Cell suspensions were prepared from both types of solid tumors and one million eosin-unstained cells were inoculated intraperitoneally into two groups containing fifteen mice each. There was no difference between the growth curves of M_{AS_1} and $M_{AS_{15}}$, and both were similar to the growth curve of one million ascites cells derived from line M_{AA} (Fig. 10).

On the other hand, the curve of M_{SS} demonstrates that the cells of the original solid line are not capable of multiplying in the peritoneal fluid after the inoculation of cell suspensions that have been prepared in the same way and contain the same number of eosin-unstained cells. It has to be emphasized that all animals inoculated with M_{SS} died eventually with large solid tumors of the peritoneal cavity and/or the abdominal wall. Usually they also had large hemorrhagic exudates which were always very poor in tumor cells.

The similarity of the growth curves of M_{AA}, M_{AS_1} and $M_{AS_{15}}$ indicates that fifteen subcutaneous passages in the solid form did not alter the ability of the ascites cells to grow in the peritoneal fluid to any measurable degree.

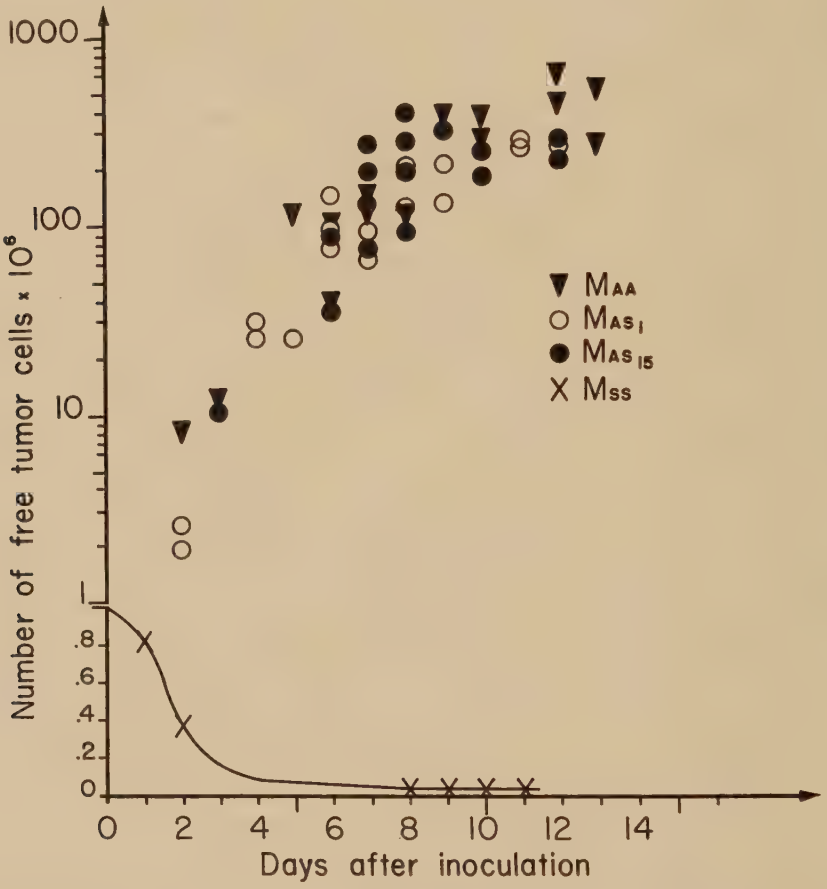

Fig. 10. Total number of free tumor cells in the peritoneal fluid after the inoculation of one million eosin-unstained cells, derived from various sublines of MC1M sarcoma as indicated. All determinations have been made by the cell-counting method. Arithmetic scale $0–10^6$, logarithmic scale $10^6–10^9$.

b) *Comparison of A_{AS_1}, $A_{AS_{21}}$ and A_{SS}.* — In analogy with the experiments reported in the previous paragraph, the corresponding solid lines of MC1A were also compared in a similar way. For each line an average of 1 million eosin-unstained tumor cells were inoculated. The resulting growth curves are

shown in Fig. 11. The growth curves of A_{AS_1} and $A_{AS_{21}}$ are similar and they both reached the same maximum tumor cell number in the peritoneal fluid. This indicates that the ability of the ascites cells to grow in the peritoneal fluid was maintained in spite of 21 solid subcutaneous passages.

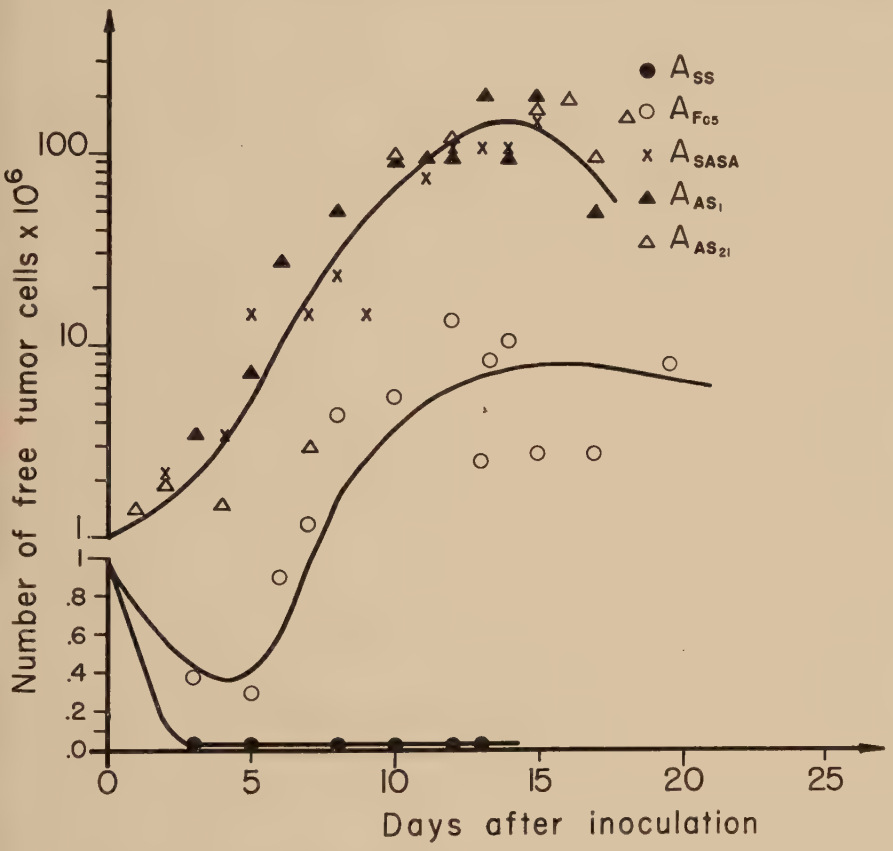

Fig. 11. Total number of tumor cells in the peritoneal fluid after the inoculation of one million eosin-unstained cells, derived from various sublines of the MC1A sarcoma, as indicated. All determinations have been made by the DNA method. Arithmetic scale 0–10^6, logarithmic scale 10^6–10^8.

A growth curve of A_{SS} is shown for comparison, indicating the early disappearance of tumor cells from the peritoneal fluid. Again it has to be pointed out that all animals inoculated with A_{SS} died eventually, showing the development of solid tumors accompanied by hemorrhagic exudates poor in tumor cells.

Transformation of A_{SS} by alternating subcutaneous and intraperitoneal transfers

As shown in Fig. 2, the ascites line (A_{AA}) of MC1A was derived from the original solid line (A_{SS}) by 24 generations of intraperitoneal exudate transfers. During the earlier part of this process, the exudates used for inoculation were secondary to the growth of solid tumors in the peritoneal cavity and contained only few tumor cells (18). The dose of inoculum is thus rather low. It has been shown, however, that even for typical ascites tumors the use of large inocula is often a prerequisite to obtain a primary multiplication of free tumor cells in the peritoneal fluid of adult mice, while small inocula give mainly rise to solid tumors (17, 18, 19). The "critical" inoculum level necessary for 100 per cent growth in A-form shows great variations with different tumor-host combinations. For A_{AA} in C3H mice it is of the order of $2–4 \times 10^5$ tumor cells. Smaller inocula lead only seldom and irregularly to typical ascites tumors and these can be usually shown to follow the ulceration of solid tumors towards the peritoneal cavity in a secondary way.

In terms of the mutation-selection hypothesis, the selective advantage of changed tumor cells possessing an increased capacity to survive and grow in the peritoneal fluid would thus be reduced by the necessity of using small inocula during the earlier part of the serial exudate transfers. If the inoculum dose could be increased, the number and consecutively the selective advantage of these cells could be expected to rise and thereby reduce the number of "fluid transfer generations" necessary to achieve the ascites transformation.

These arguments served as the theoretical basis to start the tumor line denoted A_{SASA}, the transplantation history of which appears in Fig. 2. This line originates from A_{SS} and has been carried by alternating intraperitoneal and subcutaneous transfers. In every second generation solid tumors were inoculated intraperitoneally in large amounts (0.2–0.4 g tissue per mouse). This resulted in the development of solid abdominal tumors, followed by exudate formation. The exudate, being always very poor in tumor cells, was then inoculated subcutaneously in the interposed transfer generations and regularly gave rise to solid tumors. Thus these solid tumors essentially served the purpose of enriching the few tumor cells in the exudate and to enlarge inoculum dose before every intraperitoneal transfer.

Simultaneously with A_{SASA}, a control line was started from the same inoculum ($A_{AA'}$) which was carried by serial intraperitoneal exudate transfers in the same way as the original A_{AA} line two years earlier (Fig. 2).

On a selection hypothesis, the method of alternating transfers (A_{SASA})

could be expected to reduce the number of intraperitoneal passages needed to achieve ascites transformation by increasing the selective advantage of the changed cells, as discussed above. A physiological adaptation mechanism would, on the other hand, probably be counteracted by the interposed subcutaneous transfers, permitting cell growth in the original environment for one generation after each period of exposure to the environment of the peritoneal fluid. The length of the transformation period would thus be prolonged or at least unchanged.

As appears from Fig. 2, the transformation period was actually reduced from 23 ($A_{AA'}$) to 13 (A_{SASA}) intraperitoneal transfer generations. The growth curve of the converted A_{SASA} line is closely similar to that of the established ascites line (Fig. 11). It is of importance to point out that there was good agreement between the transformation histories of A_{AA} and $A_{AA'}$.

These findings provide further indirect evidence in support of the selection hypothesis.

Serial transplantation of independent A_{SS} transfer lines started with very small inocula (FC lines)

The same suspension of A_{SS} cells that was used to start transfer lines A_{SASA} and $A_{AA'}$ (discussed in the previous paragraph) was diluted 10 times with physiological saline that had been "conditioned" (10) by injecting it into the peritoneal cavity of normal mice in 5-ml amounts and withdrawing it after 30 minutes. Such "preconditioned" saline contains protein and has been found to be useful as diluent for single-cell transfers (10, 7). The number of eosin-unstained cells in the diluted suspension was 11,250 per ml; 0.4 ml was further diluted with "conditioned" saline to a total volume of 10.4 ml; 0.01 ml of this suspension contained a statistical average of 4.3 eosin-unstained cells. This volume was inoculated subcutaneously into the right flank of 50 newborn C3H mice with an Agla micrometer syringe. Fourteen animals developed solid tumors at the site of inoculation after a latency period ranging between 34 and 63 days. Of the 14 tumors 8 (called $A_{FC_{1,\ 2,\ 5,\ 6,\ 8,\ 10,\ 11,\ 14,}}$ respectively) were established in serial transfer (Fig. 2). Although the exact number of cells which gave rise to these tumors is unknown, it is safe to say that they originated from a very small number of cells. Each line was carried by the same procedure of alternating transfers as described for A_{SASA}, consisting of intraperitoneal inoculations of solid tumor followed by subcutaneous transfers of the peritoneal exudate. Each exudate used for transfer was examined on stained smears to determine the percentage of tumor cells. As demonstrated

in Fig. 2, seven of the eight lines remained inconvertible into A-form even after having passed an average of 26 (range 21–28) intraperitoneal transfers. Line A_{SASA} can be considered as the control of the A_{FC} lines as it was started with a *large* inoculum from the same original pool of tumor cells and was carried by the same transfer procedure. It may be appropriate therefore to repeat here the fact that A_{SASA} converted to A-form after 13 intraperitoneal transfers.

One of the eight transfer lines (A_{FC_5}) converted to A-form after 27 i.p. transfers. Conversion started approximately at the 25th i.p. generation, when about 10 per cent tumor cells were present in the fluid. A growth curve made at this stage showed a certain multiplication of tumor-cells in the fluid, but their maximum number was still only one-tenth of ascites line A_{AA} (Fig. 11). By the 27th transfer, this difference disappeared and the growth curve of A_{FC_5} became similar to the other ascites lines.

Correlates of the ascites transformation

Cellular adhesiveness.—Dr. J. P. Kaltenbach has been performing metabolic studies on lines M_{SS} and M_{AS}, using cell suspensions prepared by his method (12). Preparing the suspensions, he found (13) that $35.6 \pm 3.5 \times 10^6$ cells are obtained in the final suspension from one gram solid M_{SS} tumor on an average. The corresponding figure for M_{AS} was $86.7 \pm 4.0 \times 10^6$ cells. The difference is highly significant ($P < 0.001$). The percentage of eosin-unstained cells was 21.1 ± 2.8 for M_{SS} and 30.9 ± 1.0 for M_{AS}. The probability that the difference is due to chance is $0.01 > P > 0.001$.

The question arises whether these differences reflect variation in the number of cells per gram solid tumor. DNA determinations have answered this question in the negative: M_{SS} contains 6.37 mg DNA per gram solid tumor, while M_{AS} contains 6.00 mg, indicating that cell concentration is similar in both tissues. Microscopic necrosis is not more frequent in M_{SS} than in M_{AS}, and cannot explain the higher proportion of dead cells in M_{SS} suspensions.

On microscopical examination, suspensions of M_{SS} contain a number of spindle shaped cells in addition to round cells, while M_{AS} suspensions consist predominantly of round cells (cf. Figs. 12–13). A large number of small cell clumps also occurs in M_{SS}, while M_{AS} falls apart easily into single cells. The case is similar for A_{SS} and A_{AS}. The differences in the yield of suspended cells are thus probably due to the fact that a larger number of adherent cell clumps are retained by the stainless steel mesh during the preparation of M_{SS}

suspensions than with M_{AS}. Also, adherent cells may suffer damage more easily while being forced through solid screens which may explain the higher proportion of eosin-stained, damaged cells in M_{SS}.

As cellular adhesiveness has been related to calcium content (2), A_{SS} and A_{AS} were compared and found to contain 1.45 (A_{SS}) and 1.35 (A_{AS}) μg calcium per mg dry substance, respectively. The difference is not significant.

Tumor transmission with lung tissue

A reduced cellular adhesiveness seems to be correlated with conversion to A-form, as discussed above. Since a decreased adhesiveness of tumor cells has been postulated to be an important factor in their metastasizing capacity (2), it was of interest to investigate whether conversion to A-form is followed by an increased metastasizing tendency. Experiments were performed to determine the presence of tumor cells in the lungs of mice bearing intramuscular implants of M_{SS} and M_{AS}, respectively. The technique followed was similar to that described by Goldie *et al.* (6).

M_{SS} and M_{AS} suspensions were prepared as described above and the number of eosin-unstained cells was counted. One million unstained cells were inoculated into the muscles of the right thigh of C3H mice in 0.1 ml volumes. There was no major difference in the time of appearance or growth rate of M_{SS} and M_{AS} tumors. Small intramuscular nodules were palpable by the fifth day and visible by the 7th day. On the tenth day they reached a mean diameter of about 1 cm.

The mice were killed at successive intervals, ranging between 3 and 18 days. Their lungs were removed and finely minced with scissors. The entire lung from one mouse was suspended in KRPG and injected intraperitoneally into one C3H mouse. The mice inoculated were observed for the development of solid tumors or ascites. The test was considered negative if the animals survived three months without tumor development.

The results are shown in Table I. It appears that the lungs of M_{AS}-bearing mice gave rise to tumors earlier and more regularly than those of M_{SS}-bearing animals.

Presence of myosin-like material in A_{AA} and A_{SASA}

Tumor MC1A was originally a rhabdomyosarcoma. Studying the biochemical composition of this neoplasm, Miller *et al.* (27) have found that under conditions which yielded myosin from normal muscle, a certain

TABLE I

Data on tumor transmission with lung tissue from mice bearing intramuscular M_{SS} and M_{AS} tumors

Tumor line	Age of solid intramuscular tumor in days	Number of mice inoculated with lung tissue	Number of mice developing tumors	Ratio between cols. 4/3
1	2	3	4	5
M_{SS}	5	6	0	0.0
M_{SS}	7	10	1	0.1
M_{SS}	9	6	0	0.0
M_{SS}	13	6	2	0.3
M_{SS}	18	6	6	1.0
M_{AS}	3	6	3	0.5
M_{AS}	8	15	15	1.0
M_{AS}	13	6	6	1.0
M_{AS}	14	6	6	1.0

amount of myosin-like substance could be obtained from the tumor. It was of interest to determine whether this phenomenon was specific for MC1A and whether any such material could still be obtained after the tumor was converted to A-form.

The procedure of Miller *et al.* was followed closely in preparing the myosin-like material. About 20 ml ascitic fluid was collected from mice bearing various ascites tumors, usually 3–5 animals with moderate abdominal distension for each tumor. The fluid was harvested into ice-cold centrifuge tubes. After centrifuging in a refrigerated centrifuge at 1,500 × g, the supernatant was discarded. The sediment was suspended in 75 ml of a solution containing 0.45 M KCl, 0.05 M $KHCO_3$ and 0.001 M $Na_4P_2O_7$. After homogenizing in a Waring blendor for one minute the suspension was centrifuged in a refrigerated Spinco model L ultracentrifuge (rotor no. 30) at 78,000 × g for 30 minutes to remove "heavy" material (27). A measured volume of the supernatant was dialyzed against 1,000 ml of 0.02 M KCl by stirring overnight in a cold room. Temperature was not allowed to exceed +4°C during the whole preparative procedure. A precipitate of myosin-like material was obtained from the ascites lines of MC1A, notably A_{AA} and A_{SASA}, after dialysis, while no precipitate was present if using ascites tumor cells of the MC1M, Ehrlich, Krebs 2 or Ehrlich/Landschütz lines. The precipitate was washed with 0.02 M KCl, whereafter its nitrogen content was determined and related to the nitrogen content of the entire extract. With A_{AA} it was found to contain

9.4 per cent of the total nitrogen. The corresponding figure for A_{SASA} was 7.1 per cent. Similar determinations of Miller *et al.* (27) on the original solid form of this tumor gave values between 4.7 and 13.7 per cent. These findings indicate that the ascites transformation did not result in any appreciable decrease of myosin-like material in MC1A cells.

DISCUSSION

It has been shown (16, 20) that certain mouse neoplasms are not able to grow directly in the form of freely suspended cells in the peritoneal fluid and therefore cannot be converted immediately into typical ascites tumors. Some of them are even incapable of surviving within preestablished ascitic fluids (16) and all attempts have failed to convert them into A-form. Other tumors of this category showed a better capacity for survival in preestablished exudates and could be gradually converted into A-form after a prolonged period of serial exudate transfers (18). To explain their conversion, it has been speculated (16, 18–21) that they may contain a small number of changed cells which, in contrast to the majority of the population, possess the ability to multiply under the environmental conditions of the peritoneal fluid. The serial exudate-transfer would then impose a selective pressure favoring the changed cells and conversion into A-form would be accomplished after their proportion in the cell population has reached a critical level. Arguments presented in favor of this hypothesis concerned the transplantation history of the convertible versus non-convertible tumors and the permanence of the change if once attained, even under the conditions of prolonged transfer in the solid form. These arguments are suggestive, but indirect, and do not exclude other interpretations.

The present work has re-emphasized the permanence of the change under growth conditions in the solid form and put it on a more quantitative basis by establishing the growth curves of the cell populations concerned. Furthermore, additional and more conclusive evidence has been obtained favoring the mutation-selection hypothesis. It is based on a comparison of the transplantation history of $A_{AA'}$, A_{SASA} and the A_{FC} lines, all of them having been derived from the same sample of solid MC1A sarcoma of the original A_{SS} line. A_{SASA} and the eight independent A_{FC} lines were carried by alternating i.p. inoculations of solid tumor and subcutaneous injections of peritoneal exudate. The only difference was that A_{SASA} was started with the usual large inoculum while the A_{FC} lines originated from a very small number of cells (statistical average 4.3). Thirteen i.p. transfers were needed to convert

A_{SASA} into an ascites tumor. In contrast to this, seven of the eight A_{FC} lines remained incapable of growth in the A-form despite 21–28 i.p. transfers by the same method. Only one line (A_{FC_8}) could be converted after 27 i.p. generations.

The line carried by uninterrupted intraperitoneal exudate transfer ($A_{AA'}$) converted to the A-form after 23 passages. This is in good agreement with the similar experiment carried out two years earlier (line A_{AA}) in which 24 transfer generations were needed to achieve the same result.

The following would appear as the most plausible interpretation of these findings:

The majority of the cell population in the original A_{SS} solid tumor is incapable of multiplying in the peritoneal fluid. A randomly selected small inoculum gives therefore rise to tumors that are largely inconvertible into A-form, at least within the limits imposed by the number of transfers used in the present investigation.

A relatively small number of changed cells possessing the ability to grow in the peritoneal fluid are also present in the original A_{SS} tumor. Selective overgrowth of these cells with consecutive conversion into A-form may occur after 23–24 generations of i.p. exudate transfer or after 13 generations of alternating transfer as in A_{SASA}.

The present findings are not compatible with a pure physiological adaptation, induced by the environment in the majority of the cell population. This mechanism is made improbable by the different behaviour of A_{SASA} and the A_{FC} lines, both exposed to the same environmental conditions. Furthermore, the alternating type of transfer (as with A_{SASA}) could not be expected to lead to a more rapid conversion than the continuous passage of fluid (as in A_{AA} and $A_{AA'}$), since in the former case the cells are permitted to grow in their original environment after each period of exposure to the peritoneal fluid. On the adaptation hypothesis, the conversion of A_{SASA} as compared to the other two lines could therefore be expected to be delayed or unchanged. While the results are thus in disagreement with a pure induced adaptation, they are quite compatible with the possibility that the selective advantage of the changed cells depends on their increased adaptability to the environmental conditions of the peritoneal fluid and the mechanism of conversion thus consists of the selection of adaptable mutants and their subsequent adaptation, induced by the environment.

It might be questioned whether it is necessary to assume the presence of mutants in the original A_{SS} population and whether it would not be equally possible to explain the results by assuming that they arise during the process

of transformation itself. This is made improbable, however, by the good agreement between the number of transfer generations needed to convert A_{AA} and $A_{AA'}$, separated by two years. This agreement suggests that processes such as mutation, back-mutation and possible adverse selection keep the number of changed cells at equilibrium in the A_{SS} population. The presence of changed cells in A_{SS} is also indicated by the rapid convertibility of A_{SASA}, started with a large inoculum, as opposed to the behavior of seven A_{FC} lines. The eventual transformation of A_{FC_5} is, on the other hand, most plausibly explained by assuming that mutation occurred in this line during the course of serial transfer. It is improbable that a changed cell was already included in the original population of 4 cells, as it would have been selected out very soon under such conditions, representing a large fraction of the population. In fact the opposite was true and transformation time was considerably prolonged for A_{FC_5} (27 generations) indicating that the change has taken place at a later stage.

The number of transfer generations needed to achieve transformation is comparatively high for all convertible lines of MC1A. It is actually higher than with any other convertible tumor tested previously in the same way (18). This was the main reason to choose MC1A for the present studies, as the long selection period permitted comparisons between the various sublines. The length of the selection period can be postulated to be a function of the number of mutants and the selection pressure. Both factors may differ for different tumors. The number of mutants is inaccessible to direct determinations at present, although some information may be gained from reconstruction experiments using artificial mixtures (15). Regarding selection pressure, it has to be recalled (17, 19) that even with a number of established ascites tumors, a critical minimum cell number is required in the inoculum to make growth in the A-form possible, and smaller cell numbers lead exclusively to the production of solid tumors. The exact size of the critical inoculum depends on the tumor and the genotype of the mouse (15). For M_{AA} and A_{AA}, it has been found to be between 1×10^5 and 5×10^5 tumor cells, if transplanted into F_1 hybrids of the C3H strain and one of the four other strains used in the present study. Assuming (from the A_{FC} experiments) that the changed cells represent a small fraction of the A_{SS} population, their number will be very low indeed in the small inocula used during the early generations of the transformation history of A_{AA} and $A_{AA'}$. Their number being far below critical, the changed cells cannot be expected to overgrow selectively the unchanged majority within the short span of a few passages. Their selective advantage probably consists in a longer *survival* in the peritoneal fluid which under these condi-

tions contains a random tumor-cell population mainly derived by desquamation from solid tumors that ulcerate towards the peritoneal cavity. Such differences in survival capacities of different tumor cells in the peritoneal fluid have been demonstrated for other cases (16) and can be assumed to result in a certain selective pressure. The last stage of transformation happens rapidly and abruptly (Figs. 1 and 2, see also ref. 18), the percentage of tumor cells increasing from very low to very high levels in the course of a few generations. This probably occurs after the proportion of changed cells has reached critical size in the inoculum, enabling their *multiplication* in the peritoneal fluid in contrast to the rest of the population: this would result in a sudden increase in the selective advantage of the changed cells enabling them to overgrow rapidly the original population.

The fact that selective pressure is related to inoculum dose is also demonstrated by the reduced transformation time of A_{SASA} where the interposed subcutaneous transfers make the use of large inocula possible.

The strength of the selective pressure has been subjected to further investigations, involving the determination of the number of passages necessary to convert known mixtures of M_{SS} and M_{AA} cells into A-form (15).

The question remains as to the nature of the mutational change that renders tumor-cell growth in the peritoneal fluid possible. Unchanged (SS type) and changed (AS type) cell populations show equally good growth in the solid form, their average daily linear increase being closely similar (16). The large difference between their growth characteristics under the comparatively poor (35) nutritional conditions of the peritoneal fluid suggests that the change may involve the development of a more flexible type of metabolism or/and less exacting nutritional requirements. The nutrition of solid tumor cells is highly variable in different regions (1), but at least those lying in the neighbourhood of nutrient vessels can be assumed to be under more favorable conditions than the average ascites tumor cell that receives its nourishment more indirectly from the peritoneal exudate and has to share it with 10^8–10^9 other freely floating cells. While diffusion gradients make nutritional conditions in solid tumors inhomogeneous, permitting the survival of the most adequately nourished cells on each transfer generation, ascites tumors are transmitted by cells that all receive the same and comparatively poor supply of nutrients as they float in a fluid continuously mixed by the movements of the animal and intestinal peristalsis. A parallel might exist between certain biochemical mutations of lower organisms (25) and conversion to A-form. The cells responsible for the transmission of solid tumors can be tentatively assumed as being analogous to organisms requiring well supplied

"complete media" for their growth, while the mutational change that makes growth in A-form possible may involve the development of the capacity to synthesize one or several essential metabolites which are available in the "minimal medium" of the peritoneal fluid only in limiting amounts.

Alternatively, reduction of cellular adhesiveness could be assumed to be responsible for growth in A-form. A certain difference does indeed seem to exist between the adhesiveness of M_{SS} and M_{AS} cells. Some other facts make it improbable, however, that such changes are mainly or exclusively responsible for conversion to the A-form. On the one hand, M_{SS} and A_S cells occur in the peritoneal fluid in the freely suspended form during the early stages after inoculation, but are not capable of continued multiplication there. Ascites line A_{AA} contains, on the other hand, a number of suspended cell complexes which participate in tumor-cell multiplication similarly to the free cells. The same is the case for other ascites tumors, such as the TA3 carcinoma (18) or the rat ascites hepatoma (38). Cells with considerable adhesiveness can thus still proliferate in the peritoneal fluid in the form of clumps, while others are unable to do so in spite of their occasional occurrence in the freely suspended form.

The differential transmissibility of M_{SS} and M_{AS} with lung tissue is of considerable interest. First, the validity of the detection methods may be questioned. Lungs presumably containing a small number of tumor cells were inoculated intraperitoneally in both cases. This procedure was chosen since Goldie *et al.* (6) recommend the intraperitoneal route to avoid non-specific reactions that occur frequently after the inoculation of organ brei subcutaneously or intramuscularly. Titration experiments have shown that the cell number necessary to produce 50 per cent tumors after intraperitoneal inoculation is not significantly different for M_{SS} and M_{AS} (15). Thus the differences in take-percentages after implantation of lung tissue appear to depend on a real difference in tumor cell number in the lungs.

The earlier and more frequent appearance of M_{AS} cells in the lung may have various reasons. It may represent a difference in the capacity of the intramuscular tumor to invade blood vessels, in the ability of the individual tumor cells to become detached and be carried away by the blood stream; in their power to survive in the blood; and, finally in their ability to stay, survive and multiply in the lung. Histological examination does not reveal obvious differences in the invasiveness of the two sublines. Tissue culture experiments of Schleich (30) on the same tumor lines showed, however, that M_{AS} and A_{AS} cells display a greater mobility *in vitro* than M_{SS} and A_{SS}, respectively. Furthermore, reduction of cellular adhesiveness has been

shown to be of major importance for tumor metastasis (2) and the differences between the adhesiveness of M_{SS} and M_{AS} cells may thus bear the major responsibility for their differential appearance in the lungs. Alternatively, there is some reason to believe that differences may exist in the capacity of single detached tumor cells to survive and multiply in various organs. Using cell suspensions prepared from a number of solid tumors, Watanabe (36) has found that intravenous injections do not lead to lung metastases if only discrete single cells are present in the inoculum. He obtained regular metastases when injecting suspensions containing cell clumps. On the other hand, using ascites tumors with discrete single cells, regular metastases followed intravenous inoculation in the experiments of Warren and Gates (34) and Pomeroy (29). This suggests that a difference may exist between the proliferation capacities of single isolated cells in various organs depending on whether they originate from solid or ascites tumors. Whichever might be the exact mechanism of the increased metastasizing capacity of the transformed M_{AS} subline, it suggests that changes similar to mutation may lead to increased metastasizing tendency in cells of an established tumor. It is of interest to recall in this connection that several authors such as Yamamoto (37), Essen and Kaplan (5) and Kaae (11) have found that local roentgen irradiation of various animal tumors increases their metastasizing frequency. This finding might be related to the mutagenic effect of X-rays. The possibility that cells with high metastasizing ability may be obtained by suitable selection procedures from the mixed population of a transplanted tumor, is also suggested by the experiments of Koch (23), who succeeded in obtaining a highly metastatic subline of the Ehrlich carcinoma by serially transplanting lymph node metastases.

The present investigation has re-emphasized the concept that animal neoplasms have to be regarded as highly complex mosaics of different cell types. The cells may differ in nutritional requirements, growth capacities in various tissues, adhesiveness, metastasizing ability, and, as known from the work of Hauschka and Levan and of Law, in chromosome number, immunogenetic specificity (8) and in drug resistance (24). Selective pressures imposed by varying nutritional conditions in different environments, conditions of transfer, genotype of the host, treatment with chemotherapeutics, etc., may lead to drastic changes in the characteristics of the entire population such as conversion to the ascites form, development of poly- and aneuploidy, broadening of the host range, increased metastasizability, and resistance to growth inhibitors.

SUMMARY

Further experimental evidence has been presented favoring the theory hat the gradual conversion of certain solid neoplasms into ascites tumors epresents essentially the selective overgrowth of a small number of cells vhich differ from the majority of the original population with regard to their apacity to multiply under the environmental conditions of the peritoneal luid. The change is stable and hereditary, being maintained even during rolonged propagation in the solid form.

The permanence of the change was controlled by quantitative methods neasuring the multiplication of tumor cells in the peritoneal fluid. A procedure vas developed to estimate the number of tumor cells from desoxypentosenu- leic acid determinations and was found to agree well with direct counting echniques. The growth curve of MC1A sarcoma and some other ascites umors that contain cell clumps cannot be determined by direct counting ut is accessible by the DNA method.

Eight independent transfer lines were started with very small inocula statistical average: 4.3 cells) from the original solid form of MC1A sarcoma vhich is regularly convertible to ascites form. Seven of the eight lines proved o be inconvertible under the same experimental conditions, indicating that he frequency of changed cells in the original solid population is rather low. The selective advantage of the changed cells can be increased during the rocess of gradual conversion by using large inocula.

The change that enables tumor cells to multiply in the peritoneal fluid as been found to be correlated with a reduction of cellular adhesiveness and n increased capacity to metastasize to the lungs. No changes have been found n growth rate in the solid form, nuclear size (14), calcium content, and, for he MC1A rhabdomyosarcoma, amount of myosin-like material.

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IMMEDIATE TRANSFORMATION OF SOLID INTO ASCITES TUMORS. STUDIES ON A MAMMARY CARCINOMA OF AN INBRED MOUSE STRAIN[1]

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Conversion of a solid mouse neoplasm into an ascites tumor may occur in two distinctly different ways. While some tumors can grow in the ascites form (A-form) after their first implantation into the peritoneal cavity (*immediate* transformation), others have to be carried through a series of intraperitoneal exudate-transfers before they can multiply as free cells in the peritoneal fluid (*gradual* transformation). Still other tumors cannot be converted at all.

Previous papers (11, 12) have dealt with studies on the mechanism of gradual conversion. The experimental findings could be best explained by assuming that this process is essentially the selection of a variant cell type which is present in a small proportion in the original solid tumor and is better suited than the majority for multiplication under the environmental conditions of the peritoneal fluid. The present work is concerned with the mechanism of immediate transformation. It demonstrates that the cell population of a previously inconvertible transplanted neoplasm may spontaneously acquire the ability for immediate conversion to the A-form.

MATERIALS AND METHODS

Mice.—A/St mice were used for most experiments. Breeders of this highly inbred line were received from Dr. T. S. Hauschka in 1950. The strain has been kept in our laboratory since then by 9 generations of strict brother-to-sister mating. One tumor subline has been carried in F_1 hybrids between strains A/St and C3H/St. The histocompatibility-factor requirements of the tumor lines were tested by inoculating them into F_2 hybrids produced by crossing the same two genotypes. No distinction was made between reciprocal hybrids. Some testings were also carried out with DBA/2, C57BL and C3H/St mice. All animals were kept on a standard checker diet which, as well as drinking water, was available *ad libitum* at all times. Animals of both sexes were used. Unless specified, their age was between 2 and 3 months, and their body weight 16–22 grams.

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Tumor.—A spontaneous mammary carcinoma was found in the right axillary region of a 9-month-old breeding female of the A/St strain on June 18, 1952. It had a mean diameter of about 1.5 cm, and was somewhat hemorrhagic. Histological examination showed the picture of a moderately differentiated adenocarcinoma. Its structure was variable in different areas; some parts were acinar, others consisted of solid cords and certain areas were cystic and often hemorrhagic. The tumor was finely minced with scissors, suspended in buffered physiological saline and inoculated intraperitoneally into 3 A/St and 3 A/St × C3H/St F_1 females. All animals developed solid tumors of the abdominal cavity. Two main lines were established: line *a* was consistently carried in pure A/St mice while line *b* was maintained in A/St × C3H/St F_1 hybrids. The tumor grew progressively in all mice of these two genotypes and no spontaneous regressions were noted on a material of more than 600 animals.

Methods.—Solid tumors were carried by the subcutaneous or intraperitoneal inoculation of fine tumor mince, suspended in saline. The ascites line was maintained by serial i.p. transfer of peritoneal fluid. Solid tumor growth was followed by daily caliper measurements. Three different tumor diameters were estimated and the geometric mean was calculated and plotted against time, as recommended by Schrek (23). Approximately straightline relationships were obtained on an arithmetic scale. Least square lines were fitted to the experimental points, and the regression coefficient was taken to indicate the average daily linear growth of the tumor. The growth of the ascites line was followed by determining the total number of free tumor cells, using the method of Révész and Klein (22).

To establish single-cell clones from the solid S3A tumor, cell suspensions were prepared by forcing the tissue through a 60 wires/inch stainless steel mesh, and suspending the material in Krebs-Ringer phosphate, containing 0.25 per cent gelatin (pH = 7.4). The suspension was filtered and further diluted with the same medium. The final dilution was made to contain about 500 cells per ml. Microdroplets of the size of 0.002–0.004 ml were placed on a cover slip with an Agla micrometer syringe and examined over a moist chamber for their cell content. The technique followed was the same as described by Hauschka (9). Droplets containing only one cell were picked up with a capillary micropipette, controlled by a de Fonbrune micromanipulator, at a magnification of 200 ×. Cells with smooth surface and undamaged appearance were selected. The cell was inoculated subcutaneously into the right flank of newborn A/St mice by applying air pressure from a greased syringe.

RESULTS

Transplantation history.—The main outline of the transplantation history is shown in Fig. 1. Lines *a* and *b* have been carried in a closely similar way. Solid tumor tissue was transferred intraperitoneally during the first 17 passages. This resulted in diffuse peritoneal carcinomatosis, often accompanied by the development of hemorrhagic exudates. The fluids contained only a few nucleated cells, the majority of which were of the inflammatory type. The proportion of tumor-suspected elements was very

low and never exceeded one per cent. From the 18th generation, both lines were divided into two sublines. One was carried by alternating intraperitoneal inoculations of solid tumor and subcutaneous transfers of the subsequently developing peritoneal exudate (subline SASA). In the previous

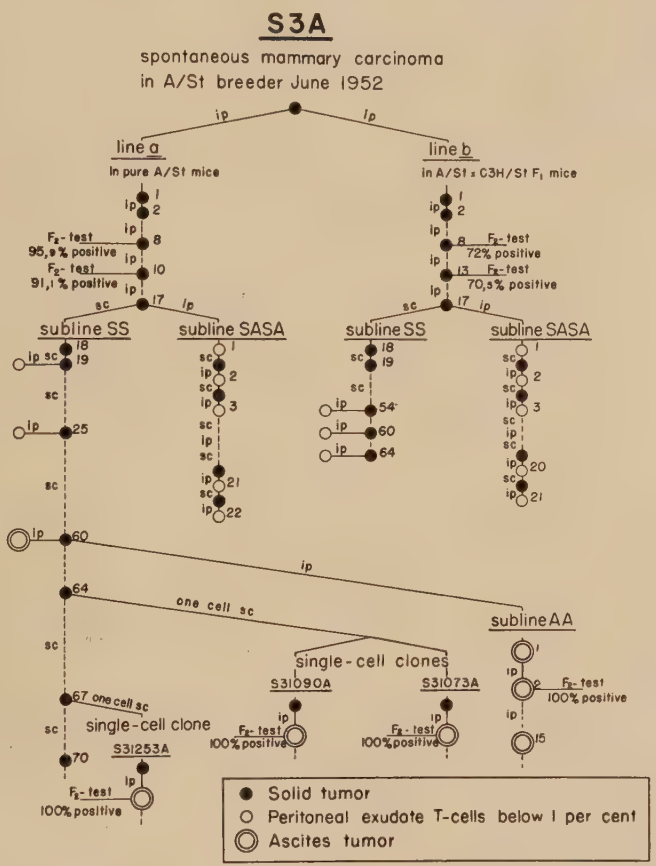

Fig. 1. Transplantation history of S3A carcinoma. The figures denote the number of serial passages. For the SASA sublines, only the number of intraperitoneal transfers is indicated.

work (12) this type of procedure was found to be useful when transforming gradually convertible solid neoplasms into ascites tumors. Altogether 22 intraperitoneal passages were made with line *a*SASA and 21 with line *b*SASA, but none of them converted to A-form. The character of the exudate did not change during the serial transfers and contained always a very small number of tumor cells only.

The two main sublines (SS-lines) were carried by subcutaneous transfer of solid tissue after the 18th generation and have until the time of the pres-

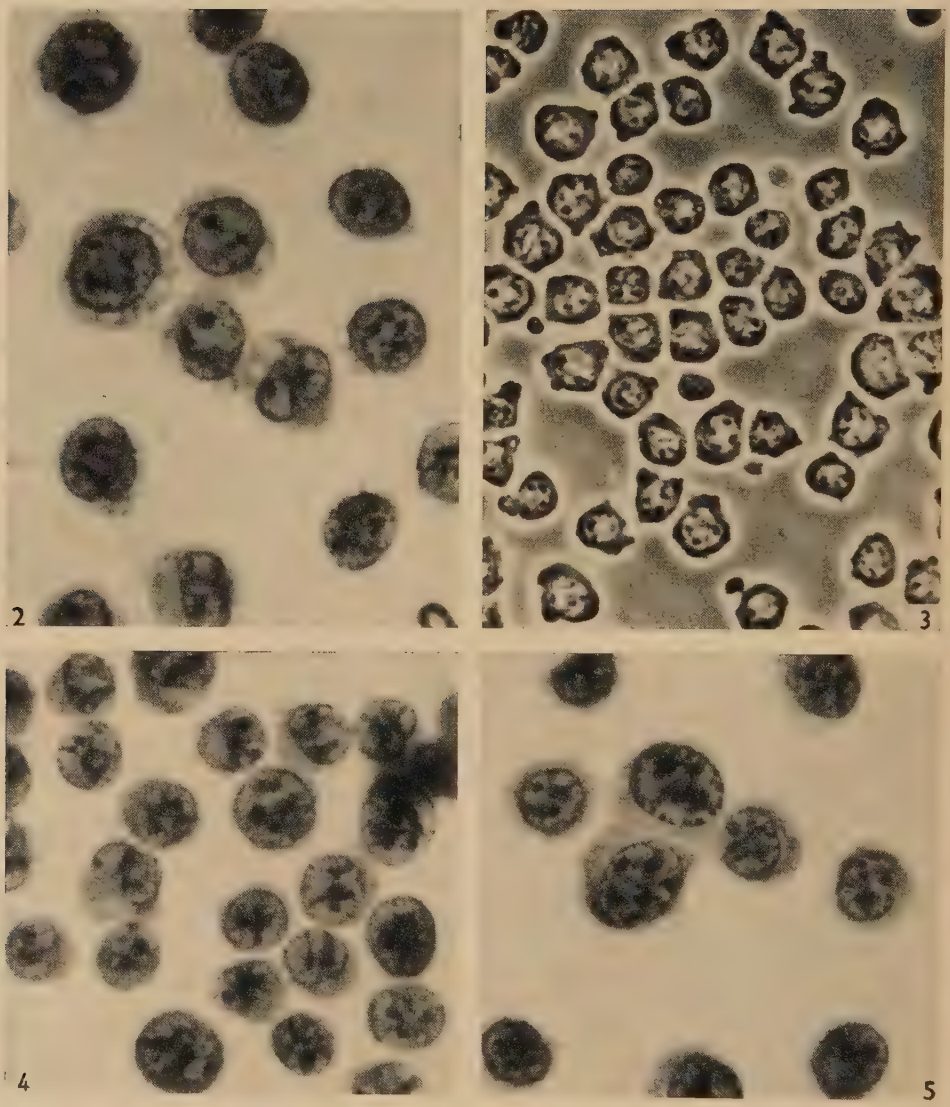

Fig. 2. Papanicolaou smear of the S3A ascites tumor. Magnification 1,200 ×.
Fig. 3. Same as Fig. 2, under phase contrast. A drop of fresh ascites was placed on a slide and slightly squashed with a cover slip. Magnification: 500 ×.
Fig. 4. Papanicolaou smear of clone S31253A. Magnification 1,200 ×.
Fig. 5. Papanicolaou smear of clone S31073A. Magnification 1,200 ×.

ent writing passed through 70 (*a*) and 64 (*b*) transfers. Line *b* remained inconvertible to A-form and was subsequently discontinued. A remarkable change occurred with line *a*, however. Tested by intraperitoneal inoculation, it was still unchanged on the 25th transfer generation. Two generations later its artificially suspended cells were tested for survival within a preestablished ascites produced by the DBA lymphoma, as reported in a previous paper (13). After a period of two days, no living S3A cells could be recovered from the fluid. No further intraperitoneal inoculations were made until the 60th transfer. On the 60th generation, one of 3 subcutaneously inoculated mice showed a largely extended abdomen. The amount of peritoneal fluid was considerably increased, had a milky white color and contained a nearly pure culture of free tumor cells. It could be shown that the subcutaneous tumor had infiltrated the peritoneal cavity by continuity. The remaining two animals of this generation were killed and their solid tumor inoculated subcutaneously into three animals in order to maintain the original solid line and intraperitoneally into ten mice. The latter gave rise in all animals to highly concentrated ascites tumors which were subsequently used to establish a special ascites transfer line (line AA, cp. Figs. 2 and 3).

Isolation of single-cell clones.—Altogether eight newborn litters, containing a total of 44 mice, were inoculated subcutaneously with a single isolated cell from the 64th and 67th transfer generations, respectively, of the solid SS line of S3A*a*. About two weeks after inoculation rapidly growing solid tumors appeared at the site of inoculation in three mice, belonging to three different litters. The rest survived an observation period of three months without tumor development. The tumorous mice were killed between the 19th and 21st day after inoculation, when their neoplasms had an average diameter of about 1 cm. The three tumor clones were named S31090A, S31073A and S31253A, resp., and cell suspensions were inoculated intraperitoneally into three groups of ten adult A mice. All animals showed the development of ascites tumors with pronounced abdominal distension and high concentration of tumor cells (Figs. 4 and 5). Part of the ascites was frozen at —79°C to preserve it for later experiments, while another part was used for F_2 tests (see below).

Growth in F_2 hybrids.—The results are shown in Table I. Lines *a* and *b* differred in their behavior when tested between the 8th and 13th transfer generation. Line *b* killed 71 per cent of the hybrids. This is not significantly different from a 3 : 1 ratio, indicating that the presence of one histocompatibility factor is required for the progressive growth of line *b* in this particular genotype. Line *a* was less specific as it was able to grow progressively

TABLE I

Growth of the various S3A lines in F_2 hybrids.

Line	Carried in	Transfer generation	Tested in	Number of mice inoculated	Number positive*	Number negative*	Per cent positive*	χ^2	P
a	A/St	8	A/St × C3H/St F_2	73	70	3	95.9	15.4	<0.001
b	A/St × C3H/St F_1	8	A/St × C3H/St F_2	74	53	21	71.6		
a	A/St	10	A/St × C3H/St F_2	45	41	4	91.1	6.9	0.01 > P > 0.001
b	A/St × C3H/St F_1	13	A/St × C3H/St F_2	64	45	19	70.3		
Combined data for line *a*	A/St	—	A/St × C3H/St F_2	118	111	7	94.0	22.5	<0.001
Combined data for line *b*	A/St × C3H/St F_1	—	A/St × C3H/St F_2	139	98	40	71.0		
Ascites line (AA)	A/St	63(3)**	A/St × C3H/St F_2	48	48	0	100.0		
Clone S31073 A	A/St	2	A/St × C3H/St F_2	30	30	0	100.0		
Clone S31090 A	A/St	2	A/St × C3H/St F_2	27	27	0	100.0		
Clone S31253 A	A/St	2	A/St × C3H/St F_2	35	35	0	100.0		

* Positive: animals dying with progressively growing tumors.
Negative: mice surviving an observation period of three months without tumor development or after their tumors had regressed.
** Total number of transfers (ascites transfers).

in 91–96 per cent. The difference is highly significant and has been found consistently on both testings. There was a decrease in the genetic specificity of line *a* after it had transformed into A-form, as it acquired the ability to grow in 100 per cent of F_2 hybrids and also in a number of foreign strains, notably C3H, C57BL and DBA/2. The same behavior was exhibited by the three single-cell clones.

The difference in the genetic specificity of the two lines was not correlated with any difference in growth rate (Fig. 6). The mean diameter of line *a* tumors increased by 0.99 mm daily on an average, the corresponding figure being 0.98 mm for line *b*. The histological picture was closely similar for both lines at the time of testing. Many signs of differentiation were still visible and the adenomatous pattern was comparatively well

preserved, although decidedly less pronounced than in the original tumor. During its later transfer generations the tumor became more and more anaplastic. By the 50th transfer both lines had lost all visible signs of differentiation and showed the picture of diffusely infiltrating round cells without any glandular pattern.

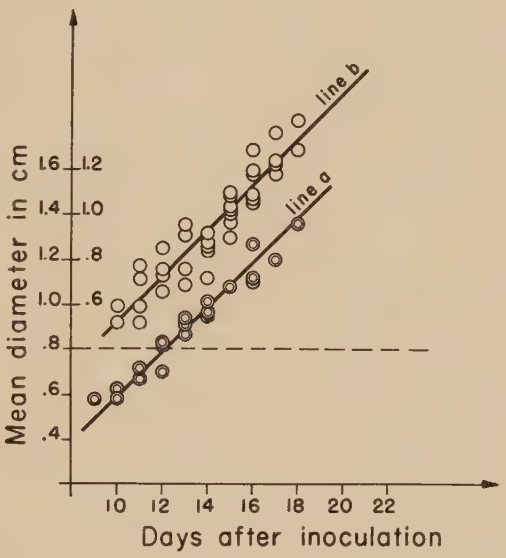

Fig. 6. Growth curve of the solid S3A tumor measured in 5 A/St (line *a*) and 10 A/St × C3H/St F_1 (line *b*) mice, respectively. The ordinate represents the geometric mean of three measured diameters for each point. The scale on the left side applies to line *a*, on the right side to line *b*. Regression equations: line *a*: $y = 0.99\ x - 0.40$; line *b*: $y = 0.98\ x - 0.46$.

Characteristics of the ascites line.—Although the conditions under which our various tumor lines are propagated were designed particularly to prevent contamination of one strain with another, it is of major importance for the present work to exclude critically the possibility of having contaminated the solid S3A tumor with cells from an established ascites tumor. The only heteroploid and genetically non-specific ascites line that was propagated at our laboratory concomitantly with S3A is the Ehrlich ascites tumor. Various characteristics of Ehrlich and the S3A ascites line were therefore compared and the results are shown in Table II. Significant differences exist in cell concentration, cell volume, mean chromosome number and size of the maximum population, indicating that one is dealing with two different tumor lines. It is of interest to point out the remarkably good correspondence between mean chromosome number and average amount of desoxypentosenucleic acid (DNA) per cell in both the Ehrlich and S3A ascites tumors.

The growth curve of one million S3A ascites cells in the peritoneal fluid

TABLE II

Characteristics of the S3A as compared to the Ehrlich ascites tumor. Figures in parentheses denote references.

	S3A	Ehrlich	P*
Average cell concentration, 10^6 per ml[1]	210.4 ± 6.6	130.9 ± 4.3 (4)	<0.001
Percentage of tumor cells	89.7 ± 1.0	86.2 ± 1.0 (18)	$0.1 > P > 0.05$
Average cell volume[1], μ^3	1 508.2 ± 74.3	1 903.0 ± 62.3 (18)	$0.01 > P > 0.001$
Modal chromosome number	72 (10)	80 (20)	
Mean chromosome number, calculated from the data of Hauschka and Levan	72.1 ± 0.2 (10)	83.2 ± 2.6 (20)	<0.001
Average DNA per cell ($\mu\mu$g)[1]	12.6 ± 0.3	14.2 ± 0.5 (4)	$0.05 > P > 0.02$
Expected average DNA value from chromosome counts[2]	12.2	14.1	
Size of the maximum population (tumor cells in millions)	700—800	1 000—2 000 (18)	
Progressive growth in C57BL mice immuniced against S3A[3]	0/7	6/8	
Progressive growth in A/St mice immunized against Ehrlich[3]	6/6	1/7	

* P: probability that the difference is due to chance.

[1] Determinations on ascitic fluids containing a total number of 100 million tumor cells or more.

[2] The calculations are based on the assumption that diploid mouse cells contain an average of 6.8 $\mu\mu$g DNA, and DNA content is proportional to chromosome number in polyploid or aneuploid cells (cp. ref. 12).

[3] Number of mice with progressively growing tumors / number of mice inoculated.

is shown in Fig. 7. It is characterized by a latency period of about two days, followed by a very rapid increase. The rate of cell multiplication decreases after a total cell number of about 250×10^6 is reached and the final maximum plateau of $700–800 \times 10^6$ is attained with continuously decreasing growth rate. There was a direct relationship between cell multiplication and the accumulation of peritoneal fluid during the majority of the growth period. The fluid was usually free of hemorrhage and infiltration of solid intraabdominal tissues was late in appearance and of limited extent. Metastasis to the lungs was frequent.

The possibility of a contamination is also excluded by experiments involving cross-immunization. C57 BL mice were immunized against S3A and A/St mice against Ehrlich in the following way: Ascitic fluid was diluted 1 : 100 with saline; 0.1 ml was injected subcutaneously into the distal part of the tail. A small tumor nodule appeared in some animals after 2–3 weeks,

which invariably regressed. After 4 weeks, 0.1 ml undiluted ascites was inoculated into the middle region of the tail. Some animals did not develop tumors, others showed incipient tumor development. The tail of the latter was cut near the base. Three weeks after the previous inoculation, 0.1 ml 1:100 diluted ascites was injected subcutaneously into the right flank. This

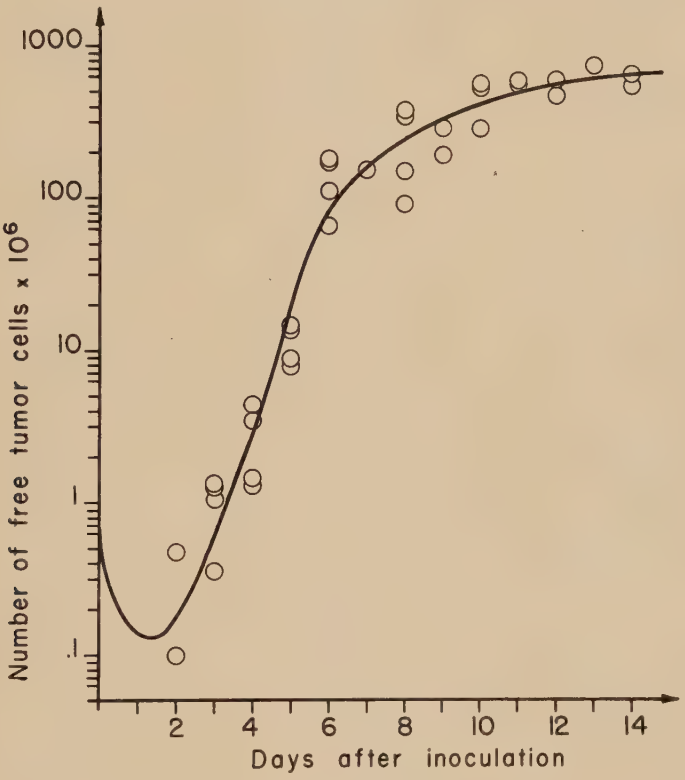

Fig. 7. Growth curve of the S3A ascites tumor. Each point represents an individual mouse.

was followed by three subsequent challenges, administered at the intervals of two weeks, and consisting of 0.1 ml 1:10 dil. fluid s.c., 0.1 ml undiluted ascites s.c., and a final challenge of 0.1 ml undiluted ascites i.p. Some mice were lost with tumors during the procedure, but the majority survived without tumors or after their tumors had regressed. Upon the last intraperitoneal challenge a number of mice developed ascites. They were punctured with glass pipettes and most of the peritoneal fluid was withdrawn. Subsequently the majority of these animals survived without further signs of tumor recurrence and proved immune to further challenges with undiluted ascites.

Three weeks after the last negative challenge the immunized mice were tested for cross immunity against the other tumor as indicated in Table II, by inoculating 0.1 ml undiluted ascites intraperitoneally. C57 BL mice immunized against S3A succumbed with Ehrlich in the majority of cases while A/St mice immunized against Ehrlich died regularly when inoculated with S3A. This proves that antigenic differences exist between the two tumors and provides further evidence against contamination.

DISCUSSION

Previous work (12, 13) was concerned with the *gradual* conversion of certain solid neoplasms into ascites tumors. No studies were made to clarify the nature of *immediate* conversion, found to occur with certain tumors after their first intraperitoneal inoculation (16). Such neoplasms as the Ehrlich carcinoma (21, 14), sarcoma 37 (6, 15), epithelioma 2146 (2) and all malignant lymphomas and leukemias tested (5–7, 15, 16, 19, 22, 24) can grow readily in A-form without a preceding series of selective passages by peritoneal exudate. It is noteworthy that all these tumors were carried in serial transplantation for a considerable period before attempts were made to convert them into A-form. A number of carcinomas and sarcomas with a short transplantation history were, on the other hand, entirely inconvertible (13, 16). Using the Ehrlich carcinoma, Löwenthal and Jahn (21) could not obtain an ascites tumor in spite of repeated trials in the course of three years. Without having altered the experimental procedure, their tumor subsequently converted to A-form in 1932.

In the present paper the transplantation history of a mammary carcinoma of the inbred A/St strain has been followed, demonstrating the development of the capacity to grow in A-form in the previously inconvertible solid tumor. The change happened somewhere between the 27th and 60th transfer generation of subline *a*SS. Three other sublines of the same tumor (*a*SASA, *b*SS and *b*SASA) that were carried a total of 60, 64 and 58 transfer generations, respectively, did not change. The possibility of contamination with another ascites tumor could be excluded.

The experiments concerning *gradual* conversion were best explained by assuming that a small proportion of altered cells have been present in the original solid tumor, possessing competitive advantage in relation to the rest of the population if exposed to the environmental conditions of the peritoneal fluid. The time needed to achieve transformation was interpreted as being necessary to shift the composition of population under deliberate

selective pressure, until a critical proportion of the variant cells is attained, enabling them to multiply in the fluid.

The spontaneous and unpredicted conversion of S3A into A-form is not necessarily due to a similar mechanism. It shows certain analogies with other abrupt changes in established tumors, denoted by the term "progression" (3), the mechanism of which is not clarified. It differs from gradual conversion by the absence of deliberately applied environmental selective pressure. If trying to explain it on similar grounds, it has to be assumed that a new cell type arose somewhere between the 27th and 60th transfer generation, possessing competitive advantage under the conditions of solid transfer and in addition also being able to grow in A-form. Its selective advantage could not be attributed to increased growth rate, as the solid line remained unaltered in this respect after the occurrence of the change. Neither was there any change in the degree of differentiation. Line *a* was highly anaplastic already before the change, but did not differ in this respect from the inconvertible *b*-line. The F_2-tests suggest a certain increase in the percentage of takes after the change (from 94 to 100 per cent) and a certain broadening of the host range was also discernible. It would be hard to conceive, however, how an increased ability to grow in foreign genotypes could lend competitive advantage to certain cells when growing in their home strain, although it may be a reflection of an increased cellular resistance against various harmful agents, among which the antibodies produced in foreign genotypes might merely be a special case.

As tentatively suggested for the case of gradual conversion, the possibility exists that the new cell type is favored because it represents a change leading from a nutritionally more dependent to a more self-sufficient type. The difference between *gradual* and *immedate* conversion would then be due to different degrees or types of change: one would lend positive selective value to the cells only under the comparatively poor nutritional conditions of the peritoneal fluid while the other would make them more adapted also for growth in the solid form.

It was questioned whether the majority of the aSS line now consists of changed cells which have overgrown the original inconvertible population. Isolation of single-cell clones has so far failed to provide evidence for the presence of the original cells since all three clones obtained grow readily in A-form and show the same high growth potentialities in F_2 hybrids as the stock tumor. This suggests that at least the majority of the present population probably consists of changed cells.

In view of the findings of Barrett and Deringer (1), Hauschka (8), and

Klein, Klein and Révész (17) concerning changes of genetic specificity after passage through F_1 hybrids, it is of interest to point out the difference between lines *a* and *b* (carried in the strain of origin and in F_1 hybrids, respectively) when tested in F_2 hybrids. Line *b* is more specific than line *a* and thus corroborates the findings of Hauschka (8), who showed that such "indirect" passage can result in an increase of genetic specificity in certain cases. In spite of this immunogenetic difference, the two lines showed the same degree of anaplasticity and had closely similar growth rates. The possible mechanisms responsible for changes occurring during transfer through hybrid genotypes have been discussed in detail (1, 8).

SUMMARY

The transplantation history of a spontaneous mammary carcinoma of the inbred A/St mouse strain has been followed through the course of serial transfer in the solid form. During the first 27 generations, the tumor was inconvertible to the ascites form. Between the 27th and 60th generation, a change occurred rendering immediate conversion to A-form possible. The change was not correlated with any alteration of growth rate or degree of differentiation and did not occur in three other lines of the same tumor. Concomitantly there was a certain decrease of genetic specificity.

Three single-cell clones isolated from the 64th and 67th generation, resp., of the solid tumor were all capable of immediate growth in A-form.

Possible contamination with the Ehrlich ascites tumor has been excluded by comparing various characteristics of the two neoplasms and demonstrating the absence of cross-immunity. In both ascites tumors, there was a good agreement between mean chromosome number and average amount of desoxypentosenucleic acid (DNA) per cell.

The tumor line maintained in the A/St strain displayed a lower genetic specificity than a subline carried in A/St × C3H/St F_1 hybrids, as measured by the percentage of progressive growth in F_2 hybrids.

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I wish to express my sincere gratitude to Dr. T. S. Hauschka for demonstrating his method for single-cell isolation at our laboratory and for communicating the unpublished results of his chromosome counts on S3A.

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Experimental Cell Research, **8**, *226–229 (1955)*

BRIEF NOTES

RECENT ELECTRON MICROSCOPE OBSERVATIONS ON THE ULTRASTRUCTURE OF THE CRAYFISH MEDIAN-TO-MOTOR GIANT SYNAPSE[1]

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Certain basic features of the crayfish median-to-motor giant synapse were described previously (6). This synapse is formed in each abdominal ganglion by the side-to-side apposition of two giant axons. It is physiologically polarized since impulses pass only from the median giant fibers to the third motor root giant fibers forming the synapse. The motor fibers extend numerous pseudopodium-like axoplasmic processes through the combined sheaths of the synapsing giant fibers. These processes measure up to 15 μ in diameter and end in median giant Schwann cytoplasm. They are completely invested by the motor fiber axon-Schwann membrane (axolemma) which measures about 300 Å in thickness by shadowing and is structurally like its median giant counterpart. Axoplasmic filaments are more concentrated in motor axoplasm than in median giant axoplasm. It is the purpose of this brief preliminary communication to present a few new observations on this synapse.

Recent electron microscope observations of very thin (c. 200–300 Å) sections through the giant synapse have revealed some principal new features which are listed below, numerically.

1) The axon-Schwann membranes in cross section consist of two dense edges with a light central zone of about 100–200 Å thickness. The complete dense edged ("double contoured") membranes measure about 200–300 Å in overall thickness. Although previously not observed (7), we now know that the motor fiber axon-Schwann membrane, which envelopes the synaptic processes of motor axoplasm fuses with the median giant axon-Schwann membrane to form a single membrane of structure apparently identical with that of either of the fusing membranes (Fig. 1).[2] The dense edges of the fusing membranes are continuous with one another as shown in

[1] This work was supported by a research grant from the National Institutes of Health, U.S. Public Health Service, from the Kansas Division of the American Cancer Society and by an institutional research grant from the American Cancer Society.

[2] Palade (4) and De Robertis and Bennett (2) reported, at the same time that our results were reported, observations on vertebrate and other invertebrate synapses similar in some respects to those described here.

Fig. 1. That these membranes are high in lipid content is suggested by the role which they and similar membranes seem to play in the metatropic reaction (3). If this role is correctly assigned, then the lipid molecules should be arranged transversely in the membranes probably in double layers whose exact location must await further evidence.

2) Concentrations of small (c. 0.1–0.8 μ), closely aggregated, concentrically lamellated bodies have been seen in motor axoplasm, particularly in the synaptic processes (Figs. 1–5). These bodies sometimes assume unusual branched shapes (Fig. 3), and the concentric lamellae of which they are composed appear, in some preparations, to consist of dense edged membranes resembling the axon-Schwann membranes (Fig. 5–6). Similar bodies appear in axoplasm in various crayfish nerve fibers and have been observed in the fibers of the circumesophageal commissures away from synapses (Fig. 6). Therefore, they are not peculiar to the synapse but merely are concentrated in great numbers in the motor axoplasm of the synaptic processes. These bodies resemble to some extent those observed by Beams and Tahmisian (1) in snail spermatozoa and by Rhodin (5) in renal epithelial cells. In the absence of specific chemical evidence these bodies cannot yet be positively identified with known cytoplasmic inclusions such as mitochondria.

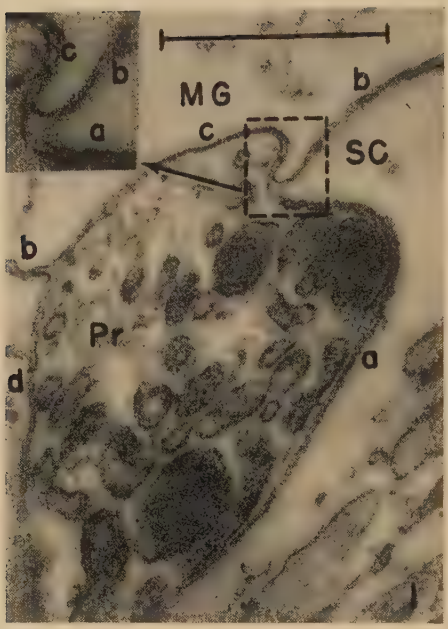
3) The axoplasm of the synaptic processes is also remarkable for numerous small vesicular bodies measuring from about 250 1000 Å in diameter. These are frequently attached to a tenuous filamentous tail around 100 Å in diameter and of indefinite length (Fig. 3). Similar vesicles are seen in median giant axoplasm and in other crayfish nerve fibers far removed from synaptic regions. However, they, like axoplasmic filaments, are greatly concentrated in the synaptic processes.

Indeed, the structural relations suggest that these vesicles may represent localized swellings of axoplasmic filaments.

Palade (4) and De Robertis and Bennett (2) have observed somewhat similar axoplasmic vesicles at vertebrate and other invertebrate synapses but they found them concentrated in presynaptic rather than postsynaptic axoplasm as we have observed. Palade (4) and the author (8) found similar vesicles concentrated in the terminal axoplasm at vertebrate myoneural junctions. However, in none of these cases has a direct association with axoplasmic filaments been noted as yet.

4) Although space does not permit its demonstration here, the synaptic processes of motor axoplasm appear to be completely surrounded at all times by a layer of Schwann cytoplasm except for the regions of fusion described above. Thus it appears that motor fiber Schwann cytoplasm fuses with median giant Schwann cytoplasm at the synapse. The relations of the outer Schwann cell membranes are not yet clear.

5) The motor fibers synapse with one another during their decussation as the median-to-motor giant synapse begins (Fig. 2). In this case all of the above synaptic features are identical. The lamellated bodies again accumulate in great numbers in the axoplasm of the processes. Although it is not shown in Fig. 2, fusion of the axon-Schwann membranes occurs in the same fashion as above. In this case, however, both synapsing giant fibers extend synaptic processes toward one another, unlike the polarized synapse with the median giant fibers in which case only the motor fibers extend such processes. It thus appears that the ability to extend axoplasmic synaptic processes is a property of the motor fibers not possessed by the median giant fibers.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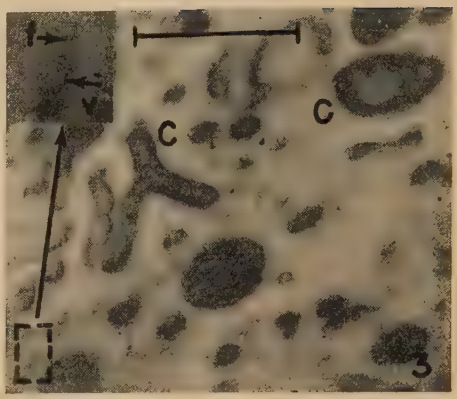
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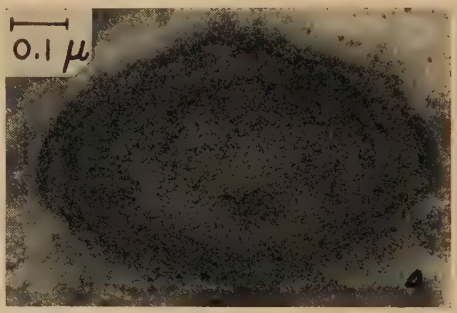
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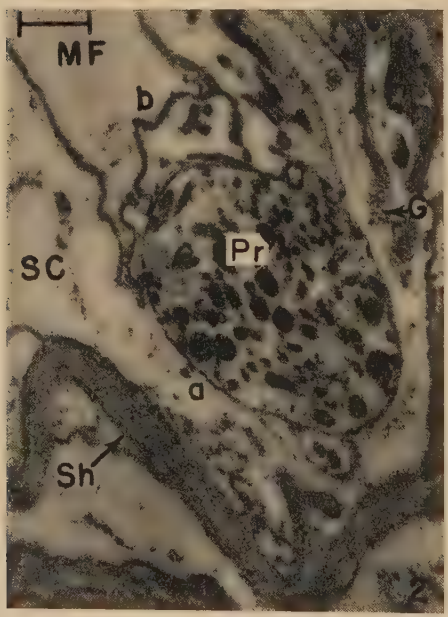
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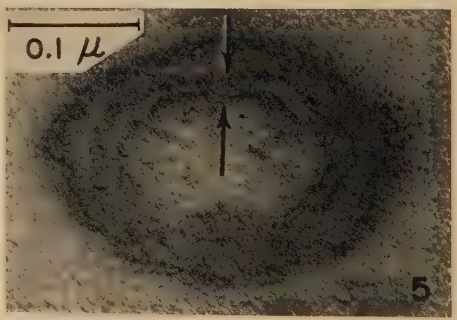
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The author wishes to acknowledge with gratitude the constant support and constructive criticism of Prof. F. O. Schmitt under whose guidance this work was begun as a Ph. D. Thesis topic.

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Fig. 1. A motor axoplasm synaptic process (Pr.) is shown in median giant Schwann cytoplasm (SC). Median giant axoplasm (MG), lies above. The motor fiber axon-Schwann membrane (a) fuses with the median giant axon-Schwann membrane (b) to produce the axon-axon synaptic membrane (c). The motor fiber axon-Schwann membrane also appears to fuse with a Schwann cell intra-cytoplasmic membrane at (d). Note the large dense bodies concentrated in the motor axoplasm of the process. These are considered to be poorly preserved concentrically lamellated bodies such as those in Figs. 3–6. The inset shows an enlargement of the dotted square. Mag. 29,000 ×. Inset mag. 59,000 ×.

Fig. 2. A motor fiber synaptic process (Pr.) containing many dense bodies is seen lying in the Schwann cytoplasm (SC) of the adjacent motor fiber at the level of motor fiber decussation. Motor fiber axoplasm (MF) lies to the upper left. The dense bodies in the process are considered analogous to those seen in the synaptic process of Fig. 1. The motor fiber axon-Schwann membranes are not fused in this locus as they are in others. An aggregated granular component of Schwann cytoplasm is seen at (G). Mag. 8,800 ×.

Fig. 3. This is an enlargement of the axoplasm of a synaptic process. Note the large (c. 0.1–0.8 μ) irregularly shaped bodies with concentric lamellation (C). A second smaller (250–1000 Å) vesicular component (v) is observed. Note the delicate filamentous strands (f) with which the latter are continuous. The inset is an enlargement of the dotted area. It shows a vesicle (v) attached to a filament (f). Mag. 20,800 ×. Inset mag. 60,500 ×.

Fig. 4. One of the concentrically lamellated bodies of a synaptic process is shown. The component membranes are poorly preserved in this case. Mag. 76,500 ×.

Fig. 5. One of the concentrically lamellated bodies of synaptic process axoplasm similar to that shown in Fig. 4 but from a different preparation is shown. The arrows designate the dense edges of one of its component concentric membranes. Only two such membranes are seen in this body. Mag. 170,000 ×.

Fig. 6. A concentrically lamellated body observed in a small nerve fiber in a circumesophageal commissure is shown. Note the resemblance of the component membranes designated by the arrows to the axon-Schwann membranes. Mag. 53,000 ×.

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ANIMALISATION DE L'ŒUF D'OURSIN PAR LES SELS DE ZINC ET DE CADMIUM

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Les travaux de Herbst (1), de Runnström (4), et de Lindahl (3) notamment, sur l'action des ions alcalins et alcalino-terreux sur le développement de l'œuf d'oursin et les remarquables anomalies morphologiques qu'ils ont mis en évidence, ont conduit aux notions maintenant classiques d'animalisation et de végétativisation. Les métaux lourds, plus toxiques n'ont pas conduit jusqu'ici, à des résultats aussi remarquables sur le développement embryonnaire. Cependant Waterman (5) a observé que la blastula de l'oursin *Arbacia punctulata*, traitée par diverses solutions de sels métalliques ne forme pas d'entoderme.

Dans cette communication préliminaire, nous nous proposons de décrire les effets des sels de zinc et de cadmium sur le développement de l'œuf de *Paracentrotus lividus*. Les œufs sont traités aussitôt après la fécondation, pendant une durée variable et par des concentrations différentes de ces sels métalliques. Les dilutions sont effectuées à partir d'une solution à 1/1.000, dans l'eau de mer. En raison du pH élevé de l'eau de mer, une partie de l'ion métallique est précipitée sous la forme d'un hydroxyde pratiquement insoluble. Cette précipitation ramène la concentration réelle de l'ion métallique à 40 % de sa valeur initiale. Les chiffres donnés ici concernent la concentration brute, sans la correction due à la précipitation d'hydroxyde métallique.

Jusqu'à la concentration 1/3.000, le chlorure de zinc bloque la segmentation à des stades variés. Quelques embryons atteignent le stade blastula, mais se lysent aussitôt. A la concentration 1/10.000, les blastulas éclosent, en retard par rapport aux témoins. Elles présentent une ciliature très développée. L'ectoderme est très épaissi et cet épaississement s'étend latéralement en direction végétative. Le mésenchyme primaire n'est représenté que par quelques cellules. L'entoderme est absent. Ces blastulas hyperciliées s'opacifient rapidement par une modification intracellulaire. L'allure de ce phénomène est particulièrement intéressante. Débutant au niveau des cellules mésenchymateuses, cette opafication gagne ensuite l'ectoderme, progressant des régions les plus végétatives vers les régions les plus animales qui sont ainsi atteintes en dernier. Les œufs traités dans ces mêmes conditions (ZnCl2, 1/10.000) pendant une durée de séjour variable, puis lavés et transférés dans l'eau de mer normale, survivent plus longtemps et montrent une série d'anomalies morphologiques intéressantes. La ciliature est très développée, correspondant dans les cas extrêmes, aux types décrits par Hörstadius (2). L'ectoderme est très épaissi sur toute son étendue. L'entoderme et le mésenchyme secondaire sont absents. L'intensité du traitement influe nettement sur la différenciation du mésenchyme primaire. Un séjour de courte durée n'entraine, en effet, qu'une altération du mode de groupement des cellules mésenchymateuses; celles-ci forment des spicules ramifiés à l'ex-

trême et enchevêtrés. Avec une altération plus accentuée, les cellules ne forment plus de spicules. La désorganisation cellulaire est marquée à un degré plus avancé par une opacification de ces cellules.

Aux concentrations plus faibles de 1/50.000 à 1/100.000, l'ectoderme forme des bourgeons dans la région la plus végétative. Ces bourgeons sont fréquemment le prolongement de replis ectodermiques latéraux. L'entoderme est représenté par une petite vésicule, ou il est absent. Les cellules mésenchymateuses sont partiellement groupées au pôle végétatif.

Aux concentrations 1/500.000 à 1/1.000.000, la caractéristique morphologique dominante est un hyperdéveloppement du lobe oral. Les bras anaux sont courts. Les spicules ramifiés forment un réseau buissonnant. L'entoderme est présent avec ses trois segments typiques, mais la bouche est absente.

Ces effets ont pu être reproduits en utilisant d'autres sels de zinc tels que le sulfate, le nitrate et l'acétate.

L'addition préalable d'un agent complexant les ions métalliques tel que le Versène 9, empêche complètement les effets de l'ion zinc sur le développement. Il est intéressant de noter ici que l'addition de Versène à des œufs préalablement traités par une solution de sel de zinc pendant une durée convenable, ne corrige pas les effets de cet ion sur le développement.

Le chlorure de lithium n'exerce qu'un effet très faible et assez irrégulier sur les anomalies morphologiques dues au traitement par les ions zinc. Cet effet ne peut d'ailleurs être observé qu'avec des concentrations en ion lithium très élevées par rapport à la concentration en ion zinc. Les modes d'action de ces ions sur le développement sont vraisemblablement différents.

Nous avons également examiné les effets du cadmium, métal aux propriétés voisines de celles du zinc. L'acétate de cadmium donne des anomalies du développement analogues à celles observées avec les sels de zinc. L'ion cadmium apparait toutefois moins actif à cet égard que l'ion zinc.

Des données expérimentales ainsi réunies, il ressort que l'ion zinc exerce un effet puissant sur le développement embryonnaire de l'œuf d'oursin. Les altérations morphologiques offrent les caractéristiques d'une animalisation typique telle que l'on peut l'observer avec les traitements animalisants classiques. La reproduction de ces effets morphologiques à l'aide de divers sels de zinc d'une part, et d'autre part, la suppression de ces effets par un agent complexant, démontrent que cet ion est l'agent actif à l'origine des animalisations observées. Il est plausible de considérer que l'embryon constitue à son tour, un système complexant, en raison de l'affinité particulière de certains de ses constituants pour les ions considérés. L'étude des modalités de la fixation de certains ions métalliques sur les constituants cellulaires, au premier rang desquels viennent se placer, mais non exclusivement toutefois, les protéines, ouvre ainsi une voie d'approche à l'analyse de l'animalisation.

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A MITOTIC RHYTHM IN THE APPEARANCE OF MITOCHONDRIA DURING THE EARLY CLEAVAGES OF THE SEA URCHIN EMBRYO

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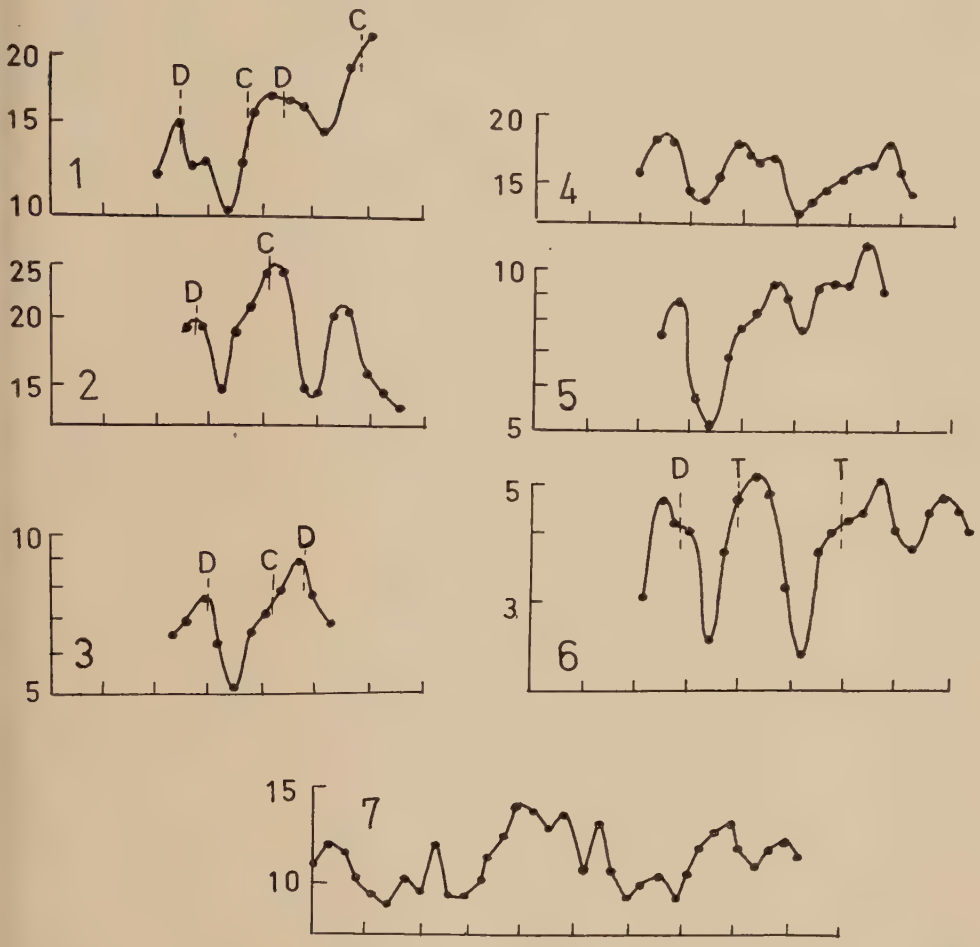
When differentiation starts in the developing sea urchin embryo an animal-vegetal gradient appears in the mitotic activity (1). A corresponding gradient is established in the appearance of mitochondria (3), the two gradients appearing at the same time but with maxima at opposite poles. Thus mitotic activity and mitochondrial formation are to a certain extent antagonistic. Nuclear action seems to be alternatively directed either to its own reorganization by mitosis or to the control of growth and differentiation of the cell, i.e. by the formation of mitochondria. In this connection it may be of interest to settle whether the single mitotic cycle is accompanied by any changes in the number of mitochondria. Thus it has been demonstrated, that the filamentous mitochondria of embryonic chicken cells in tissue culture became thinner and even fragmented during prophase and metaphase, but were reconstructed during the following phases in the mitotic cycle (2).

For this investigation embryos of the sea urchin *Psammechinus miliaris* were used. The mitochondria were observed in a phase contrast microscope at a magnification of 1.200 ×. They are seen as dark round granules about 0.2 μ in diameter very often as double granules or sometimes clustered together in larger aggregates and staining clearly with Janus Green. A faint Brownian movement can be observed. The counting of the mitochondria was started about 20 minutes after fertilization, before the nucleus entered the prophase. A sample of embryos was pipetted onto a glass slide, two human hairs were placed one on either side of the water drop onto which a cover glass was laid. Thus the embryos were slightly flattened, which is necessary to see the mitochondria clearly enough. To avoid evaporation the cover glass was surrounded by liquid paraffin. The observations were made at ordinary room temperature, but it was impossible to avoid a certain heating from the microscope lamp as shown by the higher rate of development of these embryos compared with controls in bowls on the table. For counting, an ocular square net was used. The net was adjusted above one selected embryo and the mitochondria counted along the embryo in a series of squares, each of 64 μ^2 area. By the aid of the micrometer screw the mitochondria were counted in three dimensions along a depth of 30 μ from the cell surface. Thus every count amounted to 1920 μ^3. During the presence of a spindle the centre of the cell is absolutely freed from mitochondria, which are squeezed out from the spindle. To avoid that these irregularities in the distribution should appear in the mean values the same fixed series of squares was always used, including both the central and the surrounding, more peripheral parts of the cell. Usually six squares formed one series of counts. This took about three



Diagrams 1–6. The rhythmic fluctuation in the number of mitochondria during the first cleavages in six sea urchin embryos from different samples of eggs. Horizontal axis: Developmental time. Every interval = 10 minutes. Vertical axis: Number of mitochondria per 1000 μ^3 on a logarithmic scale. D = dissolution of the nuclear membrane. C = first traces of the cleavage furrow. T = formation of telophase vacuoles.
Diagram 7. Irregular rhythm in an unfertilized egg. Denotations as before.

minutes to achieve and the same series of counts was immediately started again. Every dot in the diagrams represents the mean value of one such series.

From the diagrams 1–5 it is obvious that a rhythmic fluctuation in the mitochondrial number accompanies the mitotic cycle during the early cleavages. Mitochondria are dissolved and formed again during the mitotic period, but no regular variation in their size could be observed. In the only slightly flattened embryo it is unfortunately impossible to see the chromosomes clearly in the phase contrast

microscope and therefore it is not easy to compare the mitotic stages and the variation in the number of mitochondria directly. However, besides the formation of asters and spindle, two moments during mitosis are easily seen in phase contrast, which are also sharply defined in relation to time, *viz.*, the dissolution of the nuclear membrane and the appearance of the first traces of the cleavage furrow. The former event occurs about one minute after prophase maximum and the latter a few seconds after the separation of the chromosomes at anaphase, at which time the chromosomes suddenly become visible as bright vacuoles, in diagram 6 called telophase vacuoles, which eventually coalesce to the interphase nucleus. By combining these data and observations by sampling out at intervals and staining embryos from the same batch, it can be concluded that the number of mitochondria decreases from prophase to metaphase maximum and increases up to the next prophase. A rhythmical fluctuation in time to the mitotic cycle is seen and the maxima in mitochondrial number coincides with periods when the nucleus is whole. Thus the mitochondria formation and the mitotic activity again appear to be antagonistic.

The mitochondria represent loci for the main respiratory system of the cell, the cyclophorase system. It has been shown that the oxygen uptake of the cleaving embryo has a rhythmic fluctuation with maxima when the nuclei are whole (4). It thus seems fairly obvious that this respiratory rhythm has its cause in the established rhythmic variation with regard to the number of mitochondria. Since the oxygen uptake of the embryo steadily increases during the development, but no clear corresponding increase in the mitochondrial number is observed, it seems reasonable to presume that the embryo has two types of respiratory systems, one mitochondria bound and rhythmically varying and the other not mitochondria bound, which steadily increases in activity during the development.

In diagram 6 one embryo is represented where the cleavage and all spindle or aster formation was surpressed by the action of oestradiol. In some embryos observed, diagrams 4, 5, and the latter part of diagram 2, no dissolution of the nuclear membrane occurred, probably owing to the pressure of the cover glass, and no asters or spindles were formed. In spite of this the same mitochondrial rhythm was found to occur in all embryos. It thus seems as if the rhythmic dissolution and formation of mitochondria represented a plasmatic rhythm, which is to a certain extent independent of impulses from a dividing nucleus. In the unfertilized egg, too, a certain irregular rhythm in the number of mitochondria can be seen, diagram 7. The variation is of quite another order of magnitude than that caused by a migration of mitochondria in or out of the fixed observation squares. Even in the unfertilized egg mitochondria must thus be unceasingly formed and dissolved but according to an irregular rhythm which is later on synchronized with the mitotic rhythm at fertilization.

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ON THE EFFECT OF RIBONUCLEASE ON A JELLY PRECIPITATING FACTOR FROM THE EGG OF THE SEA URCHIN, *PSAMMECHINUS MILIARIS*

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The observations reported below were made in connection with experiments on homogenates of eggs of *Psammechinus miliaris*. These were subjected to centrifugal fractionation and the effect of the different fractions on the egg was tested. One of these fractions (Sed. II) was rich in jelly precipitating factor (JePpt F (E)) cf. (1, 6). This factor is identical with the antifertilizin of F. R. Lillie (2) and Tyler (7). It proved that addition of ribonuclease reinforced the jelly precipitating effect of the fraction.

Methods. The eggs were repeatedly washed in isotonic NaCl-solution and then homogenized in this solution by sucking the eggs through a glass filter (French pyrex E, pore size 100–120 μ). The homogenates were centrifuged for 30 minutes at 60,000 × g to remove the microscopic particles (Sed. I). The supernatant I was again spun down for 180 minutes at 105,000 × g. The sediment II obtained in this way was dispersed in isotonic NaCl-solution with the aid of a Potter homogenizer. For these experiments unfertilized as well as fertilized eggs were used. In the latter case the homogenization occurred 55 minutes after insemination. The ribonuclease preparation (Worthington Biochemical Sales Co.) was free from proteinase as shown by a very sensitive test, *viz.*, the action on the vitelline membrane of the mature unfertilized sea urchin egg. This membrane breaks down under the influence of solutions of $3 \cdot 10^{-4}$ per cent trypsin in 10 minutes (4), but was not broken down by 0.05 per cent ribonuclease. For all practical purposes it was thus free from proteolytic enzymes.

Results. Of the fractions mentioned above only the properties and the action of sediment II will be dealt with here. Sediment II was subjected to a rather thorough microscopic examination. It consisted of a fibrous birefringent material. The birefringence is positive with respect to the longitudinal direction of the fibres (1, 5). Along with the fibres there are numerous granules which appear dark in phase contrast both at low and high focusing. They represented probably agglomerates of smaller submicroscopic granules. The fibrous structures were not resolved, if the sediment II was too condensed, but the birefringence gave an indication of an ordered structure. When the sediment was allowed to swell somewhat in a 0.5 M NaCl-solution the fibrous arrangement of the sediment became conspicuous in bright field or phase contrast. A gradual dispersion occurred in the NaCl-solution at the border of the sediment particles. As a consequence of the dispersion the ordered fibrillar structure and the birefringence vanished; the granules remained but were

TABLE I A

(Kristineberg, July 26, 1954. *Psammechinus miliaris*.)

The following samples were prepared:

A 1 ml dispersed sediment II in isotonic NaCl-solution.
B 1 ml ,, ,, ,, ,, ,, ,, with 0.05 per cent cryst. ribonuclease.
C 1 ml isotonic NaCl solution.
D 1 ml ,, ,, ,, with 0.05 per cent cryst. ribonuclease.
A_u and B_u refer to sediment II-fraction from unfertilized eggs.
A_F and B_F ,, ,, ,, ,, ,, fertilized ,,
Unfertilized eggs were transferred to these different samples.

	A_u	B_u	A_F	B_F	C	D
30–40 minutes after the transfer of the eggs	Jelly coats with a precipitated rim. Strong swelling of the jelly coats	Sharply precipitated rim of the jelly coats. Strong macroscopically visible agglutination. No strong swelling of the jelly coat	No precipitation but a certain agglutination of the jelly coats. Strong swelling of these	More condensed jelly coats than in A_F but more swollen and less agglutinated than in B_u	No precipitation and no strong swelling of the jelly coats	No precipitation of the jelly coats with the exception for oocytes and some surrounding mature eggs

TABLE I B

(Kristineberg, July 26–27, 1954. *Psammechinus miliaris.*)

After about three hours the eggs in A_u, B_u, A_F, B_F, C and D of Table I a were transferred to normal sea water and inseminated.

	A_u	B_u	A_F	B_F	C	D
1–10 minutes after fertilization	Membrane rather well elevated. Eggs spherical	Membrane rather well elevated without granules. Many eggs with asymmetrical contraction and granules in the perivitelline space	Membrane well elevated. Eggs spherical	Membrane first only locally elevated but becomes more elevated after 3–4 minutes. Strong initial contraction of the egg which gradually becomes smooth	Membrane low and asymmetrical, granular. Numerous granules in the perivitelline space. Eggs not spherical	Membrane low often only locally elevated, granular. Many eggs with asymmetrical contraction
60–70 minutes after fertilization	Well elevated membrane. 2-cell stage blastomeres of normal shape	Membrane elevated. Retardation normal. 2-cell stage, separation of cells incomplete in many cases	Membrane well elevated. Retardation normal. 2-cell stage; segmentation somewhat irregular	Membrane rather well elevated. Retardation normal. 60–70 per cent 2-cell stages	Low membrane, low retardation; 2-cell stage but cells tightly pressed against each other. Separation of cells often incomplete	Membrane low. Low retardation. None or only slight furrowing of the eggs. Nuclear divisions have proceeded
25 hours after fertilization	Development to late cleavage state. Cells separated from each other	Development has advanced less than in A_u. Some few pathological gastrulae	Numerous swimming gastrulae but with pathological cell aggregates. Archenteron short	Swimming gastrulae like those in A_F but less numerous	No development to swimming larvae occurred	As in C

embedded in a non birefringent matrix. Even now no Brownian movement of the granules was observed.

The sediment II dispersed in the Potter homogenizer had a certain jelly precipitating effect. Also the supernatant II contained the JePptF but only observations on the dispersed sediment II will be considered below. The amount of dispersed sediment II corresponded to about 250 μg per ml sample.

In Table I A observations are brought together concerning the changes of the jelly coat of eggs which were subjected to sediment II dispersed in NaCl-solution. It seems that the dispersed sediment from fertilized eggs was less active than that from unfertilized eggs. The main result was, however, the effect of ribonuclease. This enzyme very strongly enhanced the precipitating effect of the dispersed sediment II. The ribonuclease alone caused no precipitation of the jelly coat, for exceptions see further below.

The dispersed sediment II also caused a strong swelling of the jelly coat. If this swelling was too rapid as in A_F no precipitated rim appeared but a certain agglutination occurred. In B_u the agglutination was still stronger and very easily demonstrable, even macroscopically.

After 3 hours the eggs were transferred to normal sea water and inseminated here, see Table I B. In C the membrane was low but cleavage occurred, whereas in D only nuclear division was observed. In A_u the development stopped in a late cleavage stage due to the dissolution of the cement keeping the cells together. This was often seen to occur under the influence of egg extracts (6). In A_F the development proceeded further but the same kind of injury occurred although it was less pronounced. B_u and B_F exhibited a more pathological development than A_u and A_F.

Several observations proved that ribonuclease is able to attack the surface of oocytes, even if this effect sometimes failed to occur owing probably to the state of the material. The enzyme was dissolved in isotonic NaCl-solution to a concentration of 0.05 per cent. In positive cases the oocytes cytolyzed under the action of this solution and the jelly precipitated around the oocyte. The JePptF was evidently released from the oocyte and diffused into the jelly coat. There was also a precipitation of the adjacent parts of the jelly coats of mature eggs surrounding the oocytes. This shows that JePptF is able to diffuse into the medium. The described changes were not observed in oocytes kept in pure NaCl-solution. In phase contrast certain small granules (< 0.5 μ) were observed as dark spots in the surface of the oocyte. These dissolved under the action of ribonuclease. They thus contained ribonucleic acid. These granules are probably aggregates of submicroscopic "microsomes". The granules were not observed to give rise to any pronounced diffraction discs as the uniform yolk granules do. The granules in question resembled much those visible when a small amount of sediment II in isotonic NaCl-solution was put between slide and cover slip and observed with phase contrast. Also the granules found in sediment II faded away under the action of ribonuclease.

Discussion. Lundblad (3) reported recently that ribonuclease causes a considerable activation of certain proteolytic enzymes in the sea urchin egg. This suggests that the enzymes in question are bound to ribonucleic acid and that their activity is inhibited as long as they remain attached to the ribonucleic acid. Upon depolymerization of the latter, active groups are released. A similar situation seems to prevail with respect to JePptF. This assumption gives a basis for the interpretation

of the observations reported above. It is of special interest that results similar to those obtained with the dispersed sediment II often emerged also from treatment of intact oocytes with ribonuclease. Granules containing ribonucleic acid are broken down and JePptF is released as evidenced by precipitation of the jelly coat of the oocyte and of adjacent eggs. Evidently ribonuclease is able to cause a pronounced cytolysis of the oocyte, whereas no obvious cytolysis occurs in the mature eggs.

The evidence presented in this as well as in Lundblad's paper (3) points to the conclusion that the ribonucleic acid inhibits the action of certain factors and enzymes in the egg.

The microsomes are considered as sites of protein synthesis in the cell. It is therefore possible that JePptF as well as certain proteolytic enzymes partly remain at their site of formation. From here they may be released gradually. Upon certain stimulations as for example subsequent to fertilization, a more pronounced release may occur. Under physiological conditions ribonuclease is perhaps not necessarily instrumental in releasing the factors tested. Recent work (8) seems to indicate that also addition of ATP facilitates the release of JePptF.

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SOME EXPERIMENTS PERTAINING TO THE CHEMICAL MECHANISMS OF THE CORTICAL REACTION IN FERTILIZATION OF SEA URCHIN EGGS[1]

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THE recent observation that an increased amount of phospholipids becomes ether-extractable (7) upon incubation of sea urchin sperm with a lipoprotein has been assumed to support the view that the cortical reaction of fertilization involves the splitting of a lipoprotein cortical complex (5, 6). It seemed therefore of interest to investigate more thoroughly this sperm-lipoprotein interaction especially in view of the possibility that through the study of such a model light may be thrown on one of the very first links in the chain of reactions leading to the activation of the egg.

The first point taken into consideration has been the kinetics of the phospholipid release when sperm are incubated with a lipoprotein.

Following the technique used in a previous investigation (7), sperm of *Arbacia lixula* were incubated at room temperature with a solution of lipovitelline, the lipoprotein of the hen's egg yolk (3) and at different time intervals the ether-extractable phospholipids were estimated (see Experimental). The results of these experiments are summarized in Table I and they show that the ether-extractable phospholipids undergo first an increase and then, upon prolonged incubation, a considerable decrease.

This suggests that at least two reactions are concurrently at work, as a result of which phospholipids are first released from the lipoprotein and then degraded with formation of ether-insoluble products.

A metabolic utilization of phospholipids by sperm has been indicated by recent work both in the case of sea urchin (10) and mammalian sperm (13). Some experiments we have done in which ether-extractable phospholipids have been estimated in suspensions of sea urchin sperm confirm these findings (Table II).

As a stage in this metabolic utilization, phospholipids may undergo an attack from phospholipases. Were this actually the case, then a hemolytic activity should appear in the course of the reaction due to the formation of lysophosphatides through the activity of phospholipase A. The data collected in Table III show that incubation of sperm with lipovitelline or egg yolk results indeed in the appearance of a hemolytic activity which, after reaching a maximum, undergoes a decrease. In most cases a second rise of hemolytic activity has been observed the significance of which

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TABLE I

μg of phospholipid-P released from lipovitelline upon incubation with sperm of *Arbacia lixula* after:

Exp. No.	Minutes							
	0	5	10	15	20	30	60	120
1	9.9	10.1	10.4	12.3	10.6	8.6		
2	7.5					8.3	7.8	7.5
3	15.5					16.0	15.0	
4	32.4		45.4		29.2			
5	29.0	35.0		22.2				
6	22.2	27.4				26.4		
7	13.8	13.8	15.4	18.8	18.1	10.7		
8	7.9	7.9	8.3	10.4	7.0	5.6		
9	7.9	11.0	10.0	8.3	10.7	9.0		
10	8.9	8.9		10.9		12.1		
11	3.0	3.5		6.3		4.5		
12	21.0	31.2		20.3				
13	7.7	7.7		8.4		7.1		

TABLE II

μg of ether-extractable phospholipid-P in sperm suspensions of *Arbacia lixula* after standing for:

Exp. No.	Minutes					
	0	5	10	15	20	30
1	10.2	5.8	5.6	5.3	4.9	4.2
2	10.7	8.4	7.5			6.9
3	9.3	7.6	6.7			6.7

is at present obscure. The fading of the hemolytic activity upon prolonged contact between sperm and substrate may be explained as due to the further attack of the lysophosphatides by phospholipase B with formation of non-hemolytic products, e.g. glyceryl-phosphoryl-choline (14). These compounds being ether-insoluble may account for the decrease of the ether-extractable phospholipids mentioned above. Investigations are in progress to check this assumption through the identification of the split-products formed during the reaction.

Control experiments on lipovitelline and egg yolk not previously acted upon by

TABLE III

Hemolytic activity (as extinction with No. 54 filter) in suspensions of sperm of *Arbacia lixula* in lipovitelline or egg yolk.

Substrate	Time of incubation, min.							
	0	5	10	15	20	30	40	60
Lipovitelline	25	91	81	48	60	68		
	45	94	133	55	178	55		
	152		163	152	117	123		
	54	202	178	238	284	258		
	69	97	112	68	69	103		
	68					113		71
Egg yolk	59	77			20	0	22	11
	56		68		38	24	47	22
	197		280		126	114	170	178
	37					124		32

sperm and on seminal fluid after removal of the sperm, have shown them to be devoid of hemolytic activity. Sperm suspensions on the other hand show a marked hemolytic activity. This fact had already been observed (2) and accounts for the hemolytic activity present in the zero time tubes.

These results thus show that sea urchin sperm are endowed with mechanisms enabling them to split lipoprotein bonds and furthermore that released phospholipids undergo a degradation first to lysophosphatides and then to other non-hemolytic products.

On the basis of these results it is tempting to speculate that the reactions just described may be extrapolated to the first steps of the activation of the egg. Namely, in the point of the entrance of the fertilizing sperm a reaction would be started involving the splitting of the lipoprotein cortical complex with release of free lipids and their further degradation to lysophosphatides.

The investigations of Öhman (9), however, indicate that during the first 10 minutes following fertilization a binding of free lipids occurs. Apart from difference in methods used for the extraction of the so-called free lipids in Öhman's and our investigations, it has to be considered that if a reaction of the kind we have described were actually limited to the cortical layer of the egg (11, 12), an increase of free lipids would hardly be detected by ordinary analytical means. Furthermore such a reaction would be expected to be a very fast one. Thus the lipid-binding observed by Öhman may be assumed to be a later occurrence. It must be emphasized indeed that our model experiment in which a very huge amount of sperm is made to react with a lipoprotein is an enormous enlargement of the reaction that normally occurs between one sperm and the surface of one egg.

The cytolytic products formed in the reaction would provide the basis for the transient cytolysis postulated by J. Loeb (4) as the first step of activation. On the other hand the present investigations indicate that the presence of lysophosphatides is also a transient occurrence as they appear to be readily transformed into non cytolytic products, so that the reaction would rapidly come to an end. How the reaction initiated at one point of the egg surface may spread all over it remains to be seen. Nor can anything be said as to how this reaction may possibly be linked to the other changes taking place in the egg upon fertilization (8, 11).

EXPERIMENTAL

Material. a) Sperm of *Arbacia lixula* were obtained by spontaneous shedding that follows cutting of the shells. They were centrifuged at about 1500 × g for 10 min. to remove excess of seminal fluid and resuspended in sea water to make a thick suspension.

b) Lipovitelline was prepared from hen's egg yolk by the method of Chargaff (3) and was finally dialysed against sea water prior to use.

Experimental procedure. a) *Estimation of liberated phospholipids.* In a typical experiment, to 2.0 cc of lipovitelline solution, 0.5 cc of sperm suspension were added in graduated glass stoppered cylinders and thoroughly mixed by gentle shaking. At various time intervals, 5.0 cc of ice-cold ether (reagent grade) were added and the mixture shaken vigorously. After standing in the cold for about 30 min. (with occasional shaking) the ether phase was carefully transferred to a test tube by means of a pipette, leaving a thin layer of it on top of the aqueous phase. The extraction was repeated three times, although preliminary experiments had shown that the third extraction removes only insignificant traces of phospholipid-P. Sometimes small aqueous drops collected at the bottom of the test tube in which case the ether extract was transferred to another tube. In a first series of experiments (7) after evaporation of the ether, the residue was dried with anhydrous Na-sulphate and redissolved in ether and chloroform. It was then found that such a procedure was superfluous and therefore after evaporation of the ether, P was determined in the residue by the colorimetric method of Allen (1). In the zero time tubes the ether extraction followed immediately upon addition of the sperm.

b) *Estimation of the hemolytic activity.* The same quantities of sperm and lipovitelline were used as above. From time to time the tubes (13 × 100 mm Pyrex test tubes) were centrifuged at about 2500 × g for 15 min., the supernatant was withdrawn and diluted 1:4 with distilled water, to make it isotonic with blood red cells. To 2.0 cc of this solution, 0.05–0.1 cc of a suspension of washed red cells was added and the mixture incubated at 37° C for 30 min. After centrifugation, the supernatant was carefully withdrawn and made up to 5.0 cc with 0.9 per cent NaCl solution. The intensity of the hemolysis was estimated from the extinction read in a Klett-Summerson photoelectric colorimeter using a 54 filter against a blank in which sea water had been substituted for sperm. In the zero time tubes centrifugation followed immediately upon addition of sperm and the mixture was not stirred. When egg yolk was used, it was diluted to about 1:5 with sea water.

SUMMARY

Upon incubation of sperm of *Arbacia lixula* with lipovitelline (the lipoprotein of the hen's egg yolk) phospholipids are released. These then appear to undergo cleavage and hemolytic products appear (possibly lysophosphatides). The hemolytic products are then also gradually removed. The bearing of these results on the problem of the activation of the egg is discussed.

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STUDIES ON VITAL STAINING OF PROTOZOA[1]

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DURING attempts at vital staining of *Paramecium*, *Loxocephalus* and *Tetrahymena*, some unexpected results were obtained. It was observed in some instances that a given concentration of Janus green B or other dyes exerts a paralysing effect on the ciliates. The unstained protozoa ceased moving through the microscopic field, though its cilia and membranellae remained beating in a disorderly way. In other experiments, instantaneous death, followed by lysis, before any visible coloration or after a faint diffuse staining of the cytoplasm, was observed.

We verified that the variation in the amount of colloidal matter in the medium was largely responsible for such differences and in the present note we report the protecting role of different colloids added to the dye solution.

Material and methods: The observations presented here are concerned with two strains of *Tetrahymena pyriformis*. One strain (axenic), GLP, was cultivated in horse manure agar plus undecorticated wheat grain in 10 ml of water. The second strain (bacterized) was maintained in the medium MMFg 30: peptone Difco 20 g; secondary sodium phosphate 2 g; liver infusion 300 ml; distilled water to 1000 ml. The pH was adjusted to 7.0. The dyes used are indicated in Table I. As protecting agents, the following substances were tried: peptone, gum acacia, trypsin, haemoglobin, methylcellulose, dextran, starch, agar-agar, alanine and cysteine.

To study the growth of *Tetrahymena* in the presence of vital dyes, we used the following culture media: gum acacia 1/100 plus Janus green 1/100,000; and also MMFg 30 plus Janus green 1/100,000. The pH was adjusted to 7.0.

The suspended protozoa were centrifuged at 1000 r.p.m. (r 10) during one minute and washed in 0.067 M phosphate buffer, pH 7.0. During the washings the original medium was diluted in steps of 1/10 until a dilution of 1/1,000,000 was reached. At this dilution the traces of collodial matter contained in the medium did not exert any detectable influence upon the process of staining.

To a small drop of fluid containing the protozoa on an uncovered microscopic slide, we added the dye solution in a ten times larger volume.

Results: Table I shows the minimal concentrations of dyes required for the immediate death of *Tetrahymena* after removal of the colloids.

Trypan red and Nyagara sky blue (Coleman) did not kill the protozoa even at much higher concentrations.

Table II shows the differences observed in survival and stainability of the protozoa when increasing dilutions of some dyes were employed.

The progressive dilution increases the intensity and sharpness of granula staining, while the diffuse staining of cytoplasm tends to become less apparent. During the

[1] This work was supported by a grant from the National Research Council of Brasil.

TABLE I

Dye	Dilution
Janus green B (G. T. Gurr)	1/200,000
Toluidine blue (G. T. Gurr)	1/100,000
Thionine (Grübler)	1/50,000
Methylene blue (Merck)	1/25,000
Brilliant cresyl blue (Merck)	1/25,000
Neutral red (Harleco)	1/1,000
Bismarck brown Y (Grübler)	1/1,000

TABLE II

Dye	Survival (in minutes)			Diffuse cyt. staining			Granula staining		
Dilution	1 / 100,000	1 / 250,000	1 / 500,000	1 / 100,000	1 / 250,000	1 / 500,000	1 / 100,000	1 / 250,000	1 / 500,000
Toluidine blue	0	10	30	++	+	−	−	+	++
Methylene blue	5	10	20	+	+	−	−	+	++
Bril. cresyl b	10	12	20	+	+	−	−	+	++
Janus green B	0	2	10			+			+
Thionine	10	30		+	−	−	+	+	++

++ indicates intense staining; + faint, and − indicates no staining at all.

time of observation the protozoa did not seem to be injured by the dye adsorbed by the granula. In successful vital staining of *Tetrahymena* the cytoplasm of the protozoa is practically clear of stain, only the granula appear stained and the motility continues normal during some time.

The addition of peptone to the dye solutions markedly changed their toxic and staining properties. A 1/1000 solution of peptone in the 1/200,000 solution of Janus green abolished the lethal effect and made possible the survival of *Tetrahymena*. A 1/2000 dilution of peptone is sufficient to abolish the lethal effect of the other dye solutions.

Gum acacia, haemoglobin, methylcellulose and agar-agar, when added to the dye solutions were also effective in protecting protozoa, but starch, dextran, alanine and cysteine were not. Trypsin is effective in abolishing the lethal effect of Janus green at 1/100,000 only at a concentration above 1/100, at pH 5.0. If the pH is raised to 7.5 (in neighbourhood of the isoelectric point), trypsin loses much of its protecting capacity.

When the protozoa are protected by these substances the digestive vacuoles are clearly visible as colored globules.

Tetrahymena can be cultivated in the media to which Janus green is added. In this case, within 24 to 48 hours, the protozoa show brilliantly stained digestive vacuoles and exhibit normal motility. These protozoa are instantaneously killed if, after successive washings, they are submitted to the same dilution of the dye in a colloid free solvent.

Ingestion of dyes adsorbed on colloidial particles apparently does not harm the protozoa, and the ingested dyes appears to be metabolised. Ball (1) made a similar observation with regard to *Paramecium.*

DFP diluted in phosphate buffer shows irreversible effects only when employed in concentrations as high as 10^{-3} M, and eserine proved to be innocuous. The histochemical methods of Koelle and Ravin for demonstration of cholinesterase were used without success. From these results it appears doubtful that the paralysing effect of the vital dyes has anything to do with the cholinesterase system reported in the papers of Seaman (4) and other workers (2 and 3).

It is to be emphasised that any quantitative data on vital staining of protozoa should take into consideration the amount of colloidal matter in the medium.

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APPAREIL DE DESHYDRATATION CONTINUE

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Le procédé de deshydratation des tissus utilisé couramment dans les laboratoires de microscopie électronique est très lent et représente une perte de temps considérable. En effet, on recommande de transvaser les prélèvements dans une série de flacons contenant des concentrations croissantes d'alcool (30 → 50 → 70 → 95 → 100–100 %, durée totale entre 3 h. et 5 h.).

Afin d'éviter les changements successifs des alcools et de garantir une deshydratation plus régulièrement progressive, nous avons construit un appareil très simple qui nous a donné de bons résultats au cours de nombreuses expériences.

En augmentant la concentration d'alcool de façon continue et régulière, de 0 à 100 %, et en évitant ainsi un changement trop brutal entre les différentes concentrations utilisées jusqu'ici, on arrive à réduire considérablement autour du tissu les courants violents du liquide observés habituellement. La structure fine des cellules deshydratées de cette manière reste bien conservée. De plus, le gain de temps est considérable.

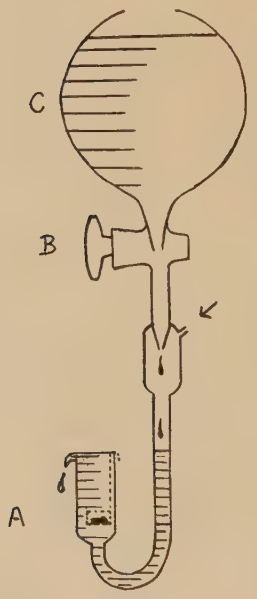

Fig. 1.

Après fixation, les morceaux de tissus sont suspendus dans le récipient A contenant le liquide de lavage (eau distillée, tampon, solution physiologique). Après 5 à 10 minutes on ouvre le robinet B et on laisse couler goutte à goutte l'alcool absolu du récipient C. Cet alcool ayant un poids spécifique inférieur à celui du liquide de A, remonte immédiatement à la surface en se mélangeant à celui-ci et en le remplaçant peu à peu. La deshydratation complète est terminée après 1 heure, mais il est possible qu'une durée plus longue soit préférable. L'écoulement de l'alcool est facilement réglable par le robinet B et contrôlé par le compte-goutte placé en-dessous. Il est important de percer une petite ouverture à ce niveau (→) afin d'assurer une pression constante à l'intérieur du tube, nécessaire à l'écoulement régulier de l'alcool.

Il est préférable de confectionner l'appareil avec du verre Pyrex. Un souffleur de verre peut le construire très rapidement en utilisant une ampoule à brome et un entonnoir cylindrique du type commercial.

Notre deshydratateur a 28 cm de hauteur, le récipient C a un volume de 150 cc, le récipient A de 20 cc (Fig. 1).

Il permet l'étude de nombreux problèmes de deshydratation aussi bien que le travail de routine de cytologie classique ou « électronique » (Fig. 2).

Fig. 2. *Foie de rat normal,* fixé 1 h. à l'acide osmique 1 % tamponné selon Palade, lavé 5' dans l'eau distillée et deshydraté en 1 h. dans l'appareil à deshydration continue. Inclusion au méthacrylate de butyle. Segment du noyau et cytoplasme avec mitochondries et éléments ergastoplasmiques. A noter la bonne conservation de la préparation. — Gross. 30.000 ×.

Experimental Cell Research, **8**, *250–251 (1955)*

THE EFFECT OF SODIUM SULPHADIAZINE ON THE NUCLEAR POPULATION OF THE PRONEPHROS IN *TRITURUS CRISTATUS KARELINII* (STRAUCH)[1]

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During the early stages of development in the Amphibia, *i.e.* blastula and gastrula stages, when the embryo has been decapsulated and suffered operative treatment, there is a liability to bacterial infection. Sodium sulphadiazine when added to the culture medium can prevent this (3), though some workers use streptomycin simultaneously as an added safeguard (7, 8). However, the chemical treatment may be responsible for effects on the development of the embryo which can only be observed by histological examination. As has been shown by Jacoby, Medawar and Wilmer (6), Jacoby (5), and Anderson, Orr and Reed (2) sulphonamides are somewhat toxic and inhibit mitosis of chick, rat and guinea pig cells *in vitro*, though sulphadiazine was found to be the least toxic. However, the effect of sulphadiazine on the numbers of cells in a single organ *in situ* during development has not yet been reported, and the following short account describes an investigation into the effects of two different strengths of sodium sulphadiazine (sodium salt of 2-(p.aminobenzenesulphonamido)-pyrimidine) on the mean nuclear population of the pronephros of *Triturus cristatus karelinii*[2], during its early development. The mean nuclear population is the average of the total nuclear populations of the right and left pronephroi in each specimen.

Three groups of larvae were immersed in their respective media at the early caudal bud stage (equivalent to H. 22–24).[3] Group I developed in N/10 Holtfreter solution alone, group II developed in N/10 Holtfreter solution plus 1.088 g of sodium sulphadiazine in a 1000 cc, and group III developed in 2.176 g in a 1000 cc. Specimens of the three groups were decapsulated between two and three days after immersion. Earlier decapsulation in the case of *karelinii* is difficult (4). Members of group I were killed at intervals between 13 and 17 days after immersion; group II, 10 to 15 days and group III 10 to 17 days. The total lengths of the members in the three groups extend between 9·75 and 14 mm.

Specimens were fixed in Smith's fixative, paraffin wax blocked, sectioned transversely at 10 microns, stained with Ehrlich's haematoxylin and counterstained with aqueous eosin. The temperature during larval development varied between 18 and 22° C. On reaching the feeding stage older specimens were supplied with liberal quantities of *Daphnia* and *Cyclops*.

The chemical treatment to which the two groups of karelinii were exposed does not significantly change the rate of development either of the total length, or nose

[1] The work was done while receiving a grant from the Agricultural Research Council, England.

[2] Generously supplied by Dr. Helen Spurway of the Dept. of Biometry, University College, London.

[3] H = Harrison series for *Amblystoma maculatum*. See Hamburger, A Manual of Experimental Embryology. Fig. 45, 1950.

TABLE I

Comparison of the three groups of *karelinii*.

	A	B	C	D	E	F	G
Group I . .	11	22	14.09±0.49	12.16±0.42	6.46±0.2	0.448±0.008	1231± 50
Group II .	8	16	13.6 ±0.62	12.34±0.66	6.25±0.18	0.456±0.01	1225± 92
Group III .	9	18	12.66±1.08	11.75±0.79	6.30±0.25	0.456±0.01	1213±112

A. Number of specimens. B. Number of pronephroi. C. Mean age in days after H 24. D. Mean total length in mm. E. Mean nose to cloacal length in mm. F. Mean antero-posterior length of pronephros mm. G. Mean nuclear population per pronephros.

to cloacal length of the larva, when these groups are compared with the controls (see Table 1). This agrees with the results of Detwiler and Robinson (3) who found no difference in the rate of development of *Amblystoma* using 1 per cent aqueous solution of the drug.

The average approximate length of 1272 pronephric tubule nuclei at right angles to the transverse section in group I is 12.45 μ; in group III 1152 nuclei averaged 12.65 μ. These data suggest that chemical treatment does not significantly alter nuclear size. An arbitrary length of 12 μ has been used to apply a correction to the pronephric nuclear populations (1). Every nucleus of the pronephric tubule seen in section by focussing up and down was counted. The first ten sections, then every other section and finally the last ten sections were counted in this way (about 70 per cent of the pronephros). Each section not counted was considered the average of the section in front and behind it. A check was made comparing the pronephric nuclear totals between three specimens of *Triturus cristatus carnifex* (a related subspecies), counting the nuclei of every section, every other section and every third section. The maximum difference was 3.5 per cent. One may assume that analysing 70 per cent of the pronephros in *karelinii* gives reliable results.

The conclusion reached is that there is a striking uniformity in the results obtained between the three groups in the pronephric nuclear populations (see Table I).

It would appear that sodium sulphadiazine at about 0.1 and 0.2 per cent and under the conditions described, has no significant effect on the mean nuclear population, or on the antero-posterior length of the pronephros during the development of *Triturus cristatus karelinii*. The treatment does not significantly change the rate of growth in total length, or nose to cloacal length.

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INHIBITION OF MUSCLE PHOSPHORYLASE BY METHYLATED OXYPURINES

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Methylated oxypurines, such as caffeine and theophylline, possess the ability to induce mutations in micro-organisms (2, 3) as well as structural chromosome changes in plant root tips (7, 8, 9). Since purines may possibly produce their cytological and genetical effects by interfering with the action of enzymes, the effect of caffeine on a number of enzymic reactions was tested *in vitro*.

As a result of these investigations it was found that the enzyme muscle phosphorylase was almost completely inhibited by caffeine in a concentration of 10^{-2} *M*. This finding was rather remarkable, since the activities of other enzymes tested, including nucleoside phosphorylase, deoxyribonuclease, xanthine oxidase, glucose-6-phosphate dehydrogenase, hexokinase, and phosphoglucomutase, were very little, if at all, affected by the same concentration of caffeine.

Methods. The phosphorylase used was prepared in crystalline form from rabbit muscle, according to the directions given by Green and Cori (4) and recrystallized three times. The activity of the enzyme was determined in two different ways, i.e., either the degradation or the synthesis of glycogen was measured. In the first case, glucose-1-phosphate, which is formed from glycogen and inorganic phosphate in the presence of phosphorylase, was converted into glucose-6-phosphate by the enzyme phosphoglucomutase. By addition of coenzyme II (TPN) and glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid was obtained. As a result of the reaction TPN is reduced, and since the reduction is accompanied by an increased absorption at 340 mμ, the process can be followed spectrophotometrically. Provided that phosphoglucomutase and glucose-6-phosphate dehydrogenase are present in excess, the increase in absorption at 340 mμ may be regarded as a measure of phosphorylase activity. The experiments were performed in 0.04 *M* tris-(hydroxymethyl)-aminomethane buffer (tris buffer), pH 6.8. The glycogen concentration normally used was 0.15 per cent.

The second method involved a colorimetric determination of the inorganic phosphate formed as a result of the phosphorylase catalyzed reaction: Glucose-1-phosphate → glycogen + phosphate. The reaction mixture had the following composition: Tris buffer, 0.1 *M*, pH 7.0; glycogen 1 per cent; cysteine, pH 7.0, 0.03 *M*; glucose-1-phosphate 0.015 *M*; inhibitor, if present, 0.0001–0.01 *M*.

Inhibition of muscle phosphorylase by purines. As mentioned, muscle phosphorylase is almost completely inhibited by caffeine in a concentration of 10^{-2} *M*. The inhibitory effect of caffeine decreased, however, with increasing concentrations of

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glucose-1-phosphate. This might indicate a competitive nature of the caffeine inhibition.

In connection with the caffeine experiments a number of other purine derivatives were also tested for their ability to inhibit muscle phosphorylase. The 8-ethers and 8-thioethers of caffeine were found to be the most effective inhibitors. They are also most active in producing chromosome breakage (8). The inhibitory action of 8-ethoxycaffeine (EOC) is roughly twice that of caffeine. Inhibition was obtained above an EOC concentration of 10^{-4} *M* and 5×10^{-3} *M* produced complete inhibition. The other purines tested, including adenine, theophylline, 1-ethyl-theobromine, 8-hydroxy-9-methyl-8.9-dihydrocaffeine (HMDC), 1.3.7.9-tetramethyluric acid, and the four possible trimethyl derivatives of uric acid, were found to be less active than caffeine. Up to a concentration of 10^{-2} *M*, practically no inhibition was obtained with adenine and HMDC.

The inhibitory effect was found to be dependent on the purine concentration during the enzymatic reaction. Pretreatment of the enzyme with higher purine concentrations in the presence of cysteine did not increase the inhibition. This seems to indicate that the purines do not produce any *permanent* changes in the phosphorylase molecule such as, for instance, a splitting of phosphorylase *a* into phosphorylase *b* (6). This conclusion is supported by the finding that dialysis of the inhibited enzyme restored activity. The dialysis experiment was performed in the following way: Two aliquots of a suspension of phosphorylase *a* crystals in cysteine -β-glycerophosphate buffer (0.03 *M*, pH 6.7) were incubated for 10 min. at 30° C with 10^{-2} *M* caffeine and water (= control), respectively. After the incubation, the mixtures were dialyzed for 3 hours at 5° C against a 0.03 *M* solution of cysteine-β-glycerophosphate (pH 6.7) under identical conditions. Activity tests showed that the activity of the caffeine treated phosphorylase was identical with that of the control both in the presence and absence of 5-AMP.

Some experiments were also performed with phosphorylase *b*. The *b*-form was obtained by incubating 200 μl of a suspension of phosphorylase *a* crystals in cysteine -β-glycerophosphate buffer (0.03 *M*, pH 6.7) for 10 min. at 30° C with 30 μl of a 0.01 per cent solution of trypsin in tris-buffer (0.1 *M*, pH 6.7). Activity tests with and without 10^{-3} *M* 5-AMP showed that phosphorylase *a* was converted into phosphorylase *b*. Since phosphorylase *b* is inactive without 5-AMP, the inhibition experiments were performed in the presence of 10^{-3} *M* 5-AMP. It appeared from the experiments that also the *b* form of phosphorylase is inhibited by caffeine.

Ability of 5-AMP to reverse inhibition. It was found that the purine inhibition of the phosphorylase catalyzed synthesis of glycogen from glucose-1-phosphate can be reversed completely by the addition of small amounts of muscle adenylic acid (5-AMP). Fig. 1 shows the effect of various 5-AMP concentrations on the inhibition produced by caffeine in a concentration of 10^{-2} *M*. An antagonistic effect was obtained with as little as 10^{-6} *M* of 5-AMP and the caffeine inhibition was completely reversed by 10^{-4} *M*.

In addition to 5-AMP a number of other natural purine compounds were tested for their ability to counteract the inhibition of phosphorylase *a* by methylated oxypurines. A slight antagonism (less than 20 per cent of inhibition reversed) was obtained with yeast adenylic acid (3-AMP) or with adenosine in concentrations equimolar to that of the inhibitor. An equimolar concentration of adenosine-5-di-

phosphate (ADP) reversed up to 60 per cent of the inhibition. Since, however, ADP increased the activity of phosphorylase *a* in the absence of inhibitors almost as much as does 5-AMP, the antagonism observed was probably due to contamination with 5-AMP. No antagonism was obtained with adenine, or with 0.1 per cent ribonucleic acid. 0.1 per cent deoxyribonucleic acid (Worthington, highly polymerized) increased inhibition and was found to be an inhibitor itself.

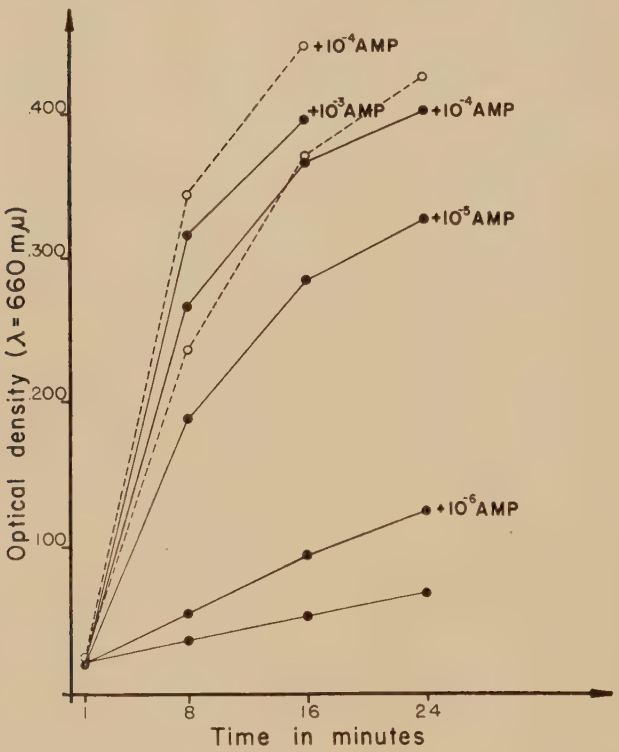

Fig. 1. The effect of adenosine-5-phosphate (AMP) on caffeine inhibition of muscle phosphorylase. The inorganic phosphate formed during reaction measured spectrophotometrically. ●———● with caffeine (10^{-2} *M*); ○- - - - - -○ without caffeine.

Thus, of the purine compounds tested, only 5-AMP can completely reverse the inhibition of muscle phosphorylase produced by caffeine and other methylated oxypurines, and most probably the antagonistic effect observed in some other cases can also be attributed to 5-AMP.

The ability of 5-AMP to counteract the inhibition of phosphorylase by methylated oxypurines seems to suggest that the antagonism is due to the structural relationship existing between inhibitor and antagonist. It must be remembered, however, that 5-AMP is to some extent also able to counteract the inhibition of phosphorylase, which is obtained with glucose or with phlorizin (1).

No purine inhibition of phosphorylases of plant origin. Since muscle phosphorylase is the only enzyme known to be completely inhibited by low concentrations of caffeine and other methylated oxypurines, and since the activities of the purines as phos-

phorylase inhibitors are, on the whole, very similar to their activities as inducers of chromosome breakage, it is tempting to see a connection between these two effects. But chromosome breakage with purine derivatives has been produced mainly in plant root tip cells. The phosphorylases obtained from plant tissues differ, however, from muscle phosphorylase in several respects of which an important one is that they do not exist in a form requiring 5-AMP for activity.

Attempts have been made to inhibit the phosphorylases in pea root tip cells and from potato with purines, but the experiments were without success. In the case of pea phosphorylase, the histochemical method introduced by Yin and Sun (10) was used for determination of phosphorylase activity. Potato phosphorylase was prepared according to Hassid, Doudoroff, and Barker (5), and the test performed *in vitro*.

Since phosphorylases of plant origin do not seem to be inhibited, there can be no direct connection between the ability of methylated oxypurines to inhibit muscle phosphorylase and their ability to produce chromosome breakage in plants. Nevertheless, the study of the mechanism underlying the inhibition of muscle phosphorylase may help us to understand the more complicated question of chromosome breakage, since both effects are probably dependent on the same properties of the purine molecule. By studying the phosphorylase inhibition by methylated oxypurines and its reversal by 5-AMP, the general function of 5-AMP in the phosphorylase action may also be better understood.

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CULTURING OF *AMOEBA PROTEUS* ON *TETRAHYMENA*

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Although *Amoeba proteus* is used extensively as an experimental cell or organism in current biological research, the techniques used for its culture are unfortunately still primitive. *A. proteus* has not yet been grown in the absence of other living organisms, which must serve as food, and the success of any culture depends to a large extent on the suitability as food of other organisms present. The usual method of setting up a stock culture consists of adding to a finger bowl a few grains of rice or wheat as the primary source of nutrient, inorganic medium, and an inoculum of amebae. Frequently, the culture is grown on an agar surface. In such an uncontrolled culture a variety of bacteria, protozoa, water molds, and sometimes rotifers and algae usually become established, and upon these the amebae feed. Extricating amebae from the mold network and washing them free of other organisms is time consuming. Because of the inhomogeniety of culture conditions, and, in particular, because food organisms are not homogeneously available, there is a wide variation in the vitality, size, and growth rate of amebae from different parts of the same culture. There are obvious disadvantages in using amebae from such cultures for many types of experiments involving single cells or small numbers of cells. Moreover, over-all growth is always slow and poor, a single culture seldom producing more than several thousand amebae.

A more satisfactory culture system has been devised in which the amebae are fed exclusively on *Tetrahymena*, which are grown separately and added to the cultures as required. There is no other source of nutrient. Control of cultures and standardization of conditions is much improved. Ameba growth is rapid and eventually becomes so heavy that amebae must be removed from the culture periodically to prevent over-crowding.

Culture Method

Inorganic medium. The inorganic medium is a new one which was designed to cover basic ion requirements and at the same time keeping the total ion concentration low. KCl, 6 mg; $CaHPO_4$, 4 mg; $MgSO_4$, 2 mg; 1000 ml of pyrex distilled water.

Food organisms. The *Tetrahymena* are grown bacteria-free in 2 per cent proteose-peptone broth. Before being fed to the amebae the *Tetrahymena* are washed twice by alternate centrifugation and resuspension in the inorganic medium.

[1] American Cancer Society Fellow, 1954—55.

Fig. 1. A small section of a young *Amoeba proteus* culture which has been without food for one hour. In the absence of food the amebae stream actively.

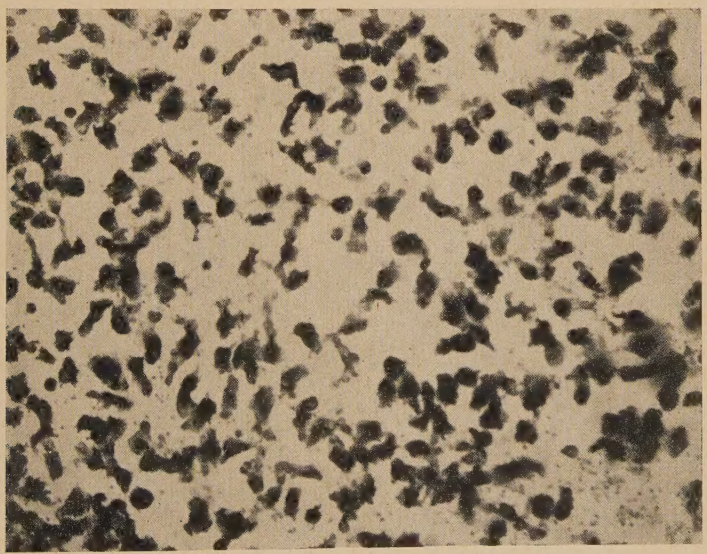

Fig. 2. A photograph of the same field as in Fig. 1 but in this case taken one minute after washed *Tetrahymena* had been added. Each ameba has taken up several food organisms and has withdrawn into a more compact form.

Amoeba culture. Cultures are maintained in finger bowls on a thin layer of 0.75 to 1.0 per cent agar and are kept in the dark at 16°C to 18°C. To initiate a culture a few millimeters of inorganic medium and a few drops of washed *Tetrahymena* are added together with an inoculum of a few thousand clean amebae. A culture can be initiated with a single ameba but naturally requires longer to develop. Enough *Tetrahymena* should be added so that there are always several within a few hundred microns of each ameba. It is preferable to add too few (thus reducing the culture growth rate) than to add too many. Since there is no nutrient available for the *Tetrahymena*, those not ingested by the amebae die off after five or six days and serve as nutrient for bacterial growth. As the bacterial population increases, ameba growth decreases. Added in the proper amount the *Tetrahymena* are used up by the amebae before the former have died. New food organisms should then be added. Fig. 1 is a photograph of a small section of a young ameba culture in which the amebae have been without food for one hour. The photograph in Fig. 2 is of the same field as in Fig. 1 but was taken one minute after washed *Tetrahymena* had been added to the culture. Each ameba has already captured several food organisms and has withdrawn into a more compact form. As long as food is plentiful and the amebae are not disturbed by light for more than a few minutes, they will remain in this form and grow and divide rapidly.

As the amebae in a culture increase in number, food organisms must be added more frequently, and after several weeks it becomes necessary to add them daily if growth is to be maintained. Culture growth can be slowed by adding food every third or fourth day or even less frequently so that the amebae are alternately growing and starving. Eventually, the culture becomes over-crowded with amebae (upwards of 100,000 in a single four-inch-diameter finger bowl), and it becomes necessary to siphon off almost all of the inorganic medium and thereby remove a large proportion of the ameba population. Growth in the culture is then renewed by adding more inorganic medium and food organisms. Old cultures can be renewed several times in this way and thus can be kept in good condition for six or seven months.

No particular effort is made to *exclude* bacteria from the cultures, but the culture dishes are kept covered as much as possible to exclude protozoa, molds, rotifers, etc. If contaminated with any of these, the culture is discarded. *Tetrahymena* are bacteria feeders and, added in the proper amount as food for the amebae, will tend to keep the bacterial population low.

It is hoped that eventually *A. proteus* can be grown under completely controlled conditions. The method of culture described here represents a step in this direction, eliminating some of the undesirable characteristics of the rice type culture. In initiating a new culture, growth immediately reaches the maximum level. Cultures are clean and large numbers of amebae can be quickly and easily washed. The ameba yield per culture is high. Because food organisms are of a single type and are homogeneously available, the amebae form a physiologically uniform population. The culture is really single-membered since the *Tetrahymena* cannot grow but only serve as food.